UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Nitrogen and metals as multiple stressors affecting the auto-remediation role of salt marshes: consequences to the ecosystem services

Ana Isabel Francisco Sousa

DOUTORAMENTO EM BIOLOGIA

(ECOLOGIA)

2010

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Nitrogen and metals as multiple stressors affecting the auto-remediation role of salt marshes: consequences to the ecosystem services

Ana Isabel Francisco Sousa

Tese orientada por:

Doutora Ana Isabel Lillebø

Prof. Doutora Isabel Caçador

DOUTORAMENTO EM BIOLOGIA

(ECOLOGIA)

2010

"ecology"(1870): *"the body of knowledge concerning the economy of nature-the investigation of the total relations of the animal both to its inorganic and to its organic environment including above all, its friendly and inimical relations with those animals and plants with which it comes directly or indirectly into contact-in a word, ecology is the study of all those complex interrelations referred to by Darwin as the conditions of the struggle for existence."*

Ernst Heinrich Haeckel

(1834-1919)

Doctoral dissertation in Biology
(specialization in Ecology)
presented to the University of Lisboa

Dissertação apresentada à
Universidade de Lisboa para obtenção
do grau de Doutor em Biologia
(especialidade Ecologia)

Ana Isabel Francisco Sousa

2010

Declaração

Para efeitos do disposto nº2 do Art. 8º do Dec-Lei 388/70, o autor da dissertação declara que interveio na concepção do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados e submetidos para publicação.

Ana Isabel Francisco Sousa

Setembro de 2010

This thesis is based on the following manuscripts:

Sousa, A.I., Lillebø, A.I, Pardal, M.A., Caçador, I., 2010. Productivity and nutrient cycling in salt marshes: contribution to ecosystem health. Estuarine, Coastal and Shelf Science 87, 640-646.
DOI:10.1016/j.ecss.2010.03.007

Sousa, A.I., Lillebø, A.I., Caçador, I., Pardal, M.A., 2008. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628-635.
DOI:10.1016/j.envpol.2008.06.022

Sousa, A.I., Lillebø, A.I., Risgaard-Petersen, N., Pardal, M.A., Caçador, I. Denitrification in *S. maritima* salt marshes: a contribution to reduce eutrophication as a service provided in salt marshes. (Under review in Marine Ecology Progress Series).

Sousa, A.I., Caçador, I., Lillebø, A.I., Pardal, M.A., 2008. Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites. Chemosphere 70, 850–857.
DOI:10.1016/j.chemosphere.2007.07.012

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I. Influence of multiple stressors on the auto-remediation processes occurring in salt marshes. (Submitted to Marine Pollution Bulletin).

Sousa, A.I., Lillebø, A.I., Risgaard-Petersen, N., Pardal, M.A., Caçador, I. Denitrification in salt marshes with different historical metal contamination: comparison of two temperate estuaries. (In preparation).

Sousa, A.I., Lillebø, A.I., Risgaard-Petersen, N., Pardal, M.A., Caçador, I. Salt marshes' meaning on nitrogen remediation, In: Bioremediation: Biotechnology, Engineering and Environmental Management, Frank Columbus (chief ed.) Nova Science Publishers, Inc. NY, USA. (Accepted for publication).

CONTENTS

ABSTRACT

The fast increase of human population and activities during the 20th century led to an increment in the loading of both land-derived nitrogen from anthropogenic diffuse sources and metal industrial discharges to coastal and transitional waters. Thus, estuaries were subdued to large discharges of nitrogen and metals, which may lead to eutrophication and historical contamination. Salt marshes provide crucial ecosystem functions, such as nitrogen cycling and sequestration, as well as phytoremediation. Therefore, this thesis focuses on a better understanding of nitrogen cycling in warm-temperate salt marshes, metal compartmentalization in salt marsh plants and effects of multiple stressors (nitrogen enrichment and metal historical contamination) on the ability to auto-remediate estuarine systems. Nitrogen sequestration and cycling in salt marshes, namely through nitrogen incorporation in biomass and organic nitrogen burial, is species-specific (*Sarcocornia fruticosa, Sarcocornia perennis, Halimione portulacoides, Scirpus maritimus* and *Spartina maritima*) and greatly depends on the maturity of the salt marsh (*S. maritima*), rather than on the estuary. Denitrification occurring in *S. maritima* salt marshes is also an important remediation process for nitrogen, namely during winter. *H. portulacoides* ability to accumulate high metals concentrations is higher in the roots than in the aboveground material and metal compartmentalization mostly occurs in the cell wall, thus, outside key metabolic sites. Regarding the studied multiple stressors, nitrogen loading and metals contamination did not affect the phytoremediation capacity of *H. portulacoides* for Zn, Cu and Ni, and enhanced the Cd accumulation in this plant species. Denitrification in metals-contaminated salt marsh was higher during the studied season (winter), when compared to a non-contaminated salt marsh. As a whole, multiple stressors affected the auto-remediation capacity of salt marshes. Since ecosystem functions seem to be species-specific, one cannot exclude that multiple stressors threaten the provided ecosystem services and, consequently, ecosystem health and equilibrium may be endangered.

Keywords: eutrophication; metal contamination; multiple stressors; salt marshes; ecosystem services

RESUMO

Ao longo do século XX, o aumento da população humana nas zonas costeiras e o aumento da pressão exercida no ambiente, inerentes à sua presença e actividades, aumentaram de forma muito rápida, o que conduziu a um grande aumento de descargas azoto de fontes difusas e origem antropogénica, tal como ao aumento de descargas de resíduos industriais (e.g. metais) para as massas de águas marinhas e de transição. Deste modo, os estuários foram sujeitos a grandes descargas de azoto e de metais, cujas consequências são de ordem diversa, nomeadamente eutrofização e contaminação por metais, denominadas, respectivamente, por eutrofização cultural e contaminação histórica por metais. Os sapais desempenham funções ecológicas extremamente importantes, nomeadamente como bio-estabilizadores; também constituem um dos ecossistemas mais produtivos e prestam serviços muito importantes, tais como recliclagem e sequestro de azoto e fitoremediação. Tendo em conta a importância destes ecossistemas, o presente trabalho tem por objectivo estudar e compreender o ciclo do azoto nos sapais; a compartimentação dos metais nos diferentes órgãos das plantas de sapal, e ainda os efeitos dos stresses múltiplos (enriquecimento em azoto e contaminação histórica por metais) na capacidade de auto-remediação dos sapais, bem como os efeitos nos serviços prestados por estes ecossistemas.

O ciclo do azoto nos sapais foi estudado por meio de 3 casos de estudo incluídos no Capítulo I.

Este trabalho consistiu na monitorização bimensal da biomassa e concentração de azoto nos diferentes órgãos das halófitas de sapal e no rizosedimento. De acordo com os resultados obtidos, a acumulação/sequestro e transferência de azoto pelas plantas de sapal é específica para cada espécie (nomeadamente, *Sarcocornia fruticosa, Sarcocornia perennis, Halimione portulacoides, Scirpus maritimus* e *Spartina martitima*) e não foi possível estabelecer uma relação entre a capacidade de retenção de azoto e a respectivo mecanismo fotossintético. Este trabalho demonstra que os processos de reciclagem de azoto, promovidos pelas plantas de sapal, contribuem para a redução da eutrofização (via sequestro de azoto), evidenciando os serviços prestados por estes ecossistemas e o papel crucial das halófitas na manutenção das funções e da saúde do ecossistema.

Através da monitorização da biomassa de *S. maritima*, conteúdo em azoto na planta, nos detritos e no sedimento, este trabalho permitiu concluir que o sapal mais maturo e que está sujeito uma pressão antropogénica superior, apresenta maior produção de biomassa e

produção de azoto na parte subterrânea da planta. Apresenta também uma taxa de decomposição mais lenta, contribuindo deste modo para um maior sequestro de azoto no sedimento. Sapais sujeitos a uma pressão antropogénica menos intensa produzem maior biomassa (e incorporam maior quantidade de azoto) na parte aérea da planta. Os resultados deste caso de estudo permitem concluir que a capacidade de retenção de azoto depende, de forma determinante, da maturidade do sapal em que se insere, bem como das características fisico-químicas inerentes. Além disso, este trabalho realça o facto de as funções prestadas pelos sapais, nomeadamente o sequestro de N, contribuírem para a redução da eutrofização em águas de transição.

Foi realizado um estudo sazonal num sapal colonizado por *S. maritima* e na área adjacente sem vegetação. Através da quantificação de fluxos de oxigénio, NH_4-N, NO_x-N, nitrificação potencial e desnitrificação ("^{15}N-isotope pairing technique"), observou-se que as taxas de nitrificação potencial foram significativamente superiores no outono e no inverno e que não houve diferenças significativas entre os dois tipos de sedimento analisados: sedimento não colonizado e sedimento colonizado por *S. maritima*. As taxas de desnitrificação em sedimentos sem vegetação (máx. 151±24 μmol $N_2m^{-2}h^{-1}$ (média ± DP) (verão, período nocturno)) estão compreendidas nos intervalos de valores obtidos noutros sistemas comparáveis. As taxas de desnitrificação no sedimento colonizado por *S. maritima* foram de modo geral superiores aos valores obtidos para a lagoa de Veneza. Relativamente à sazonalidade, as taxas de desnitrificação apresentaram valores superiores no inverno também no período nocturno (676 ± 497 μmol $N_2m^{-2}h^{-1}$) (média ± DP). No estuário deste caso de estudo, o estuário do Tejo, a desnitrificação nos sapais de *S. maritima*, quando comparadas com as obtidas na zona sem vegetação, apresentaram valores superiores no inverno. Este processo pode contribuir potencialmente para uma grande redução da concentração de azoto no estuário do Tejo nesta estação do ano (devido ao aumento da pluviosidade, das descargas fluviais e da escorrência superficial), contribuindo para a redução da disponibilidade de nitrato na coluna de água na primavera seguinte.

O Capítulo II diz respeito à acumulação e compartimentação de metais em halófitas de sapal, tendo como caso de estudo a halófita *Halimione portulacoides*.

Tendo em conta a elevada capacidade dos sedimentos colonizados por plantas de sapal para acumular elevadas concentrações de metais, e consequentemente a capacidade das plantas para tolerarem estas mesmas concentrações, este estudo teve por objectivo esclarecer quais as estratégias de *H. portulacoides* para evitar a toxicidade por metais nos diferentes

órgãos e ao nível da célula. Neste sentido, foi realizada uma extracção sequencial ao nível da folha, caule e raiz de *H. portulacoides* e determinadas as concentrações de metais (Zn, Pb, Co, Cd, Ni e Cu) em diferentes fracções do material vegetal.

De acordo com este estudo, todos os órgãos da planta acumulam os metais maioritariamente na parede celular (53 % nas folhas a 65 % nas raízes) sendo o conteúdo a nível intracelular consideravelmente inferior (21% nas raízes a 32% nas folhas). Deste modo, as concentrações metais elevadas existentes no ambiente sedimentar não causam toxicidade às plantas de sapal. Isto deve-se ao facto destas imobilizarem os metais em diferentes compartimentos celulares (parede celular, fracção proteica e intracellular) e fora de locais vitais em termos metabólicos, o que deverá ser crucial para a sobrevivência de *H. portulacoides* em sapais com elevada contaminação por metais.

No Capítulo III é abordado de duas formas distintas, os efeitos dos stresses múltiplos. i.e., excesso de azoto e contaminação por metais, na capacidade de auto-remediação dos sapais e consequentes ameaças para os serviços prestados por estes ecossistemas.

No intuito de compreender melhor como é afectada a capacidade de fitoremediação das plantas de sapal (fitoacumulação de metais) pela "eutrofização cultural", foi realizada uma experiência sob condições controladas. A contaminação histórica foi simulada expondo as plantas (*H. portulacoides*) a elevadas concentrações de metais (Zn, Cu, Ni, Cd) e foram testados diferentes níveis de enriquecimento em azoto na forma de nitrato, de modo a simular diferentes níveis de eutrofização. De acordo com o presente trabalho, e tendo em conta as condições testadas, a "eutrofização cultural" parece não afectar a capacidade de fitoremediação de Zn, Cu e Ni por *H. Portulacoides*. Mais, o serviço de fitoremediação de Cd parece ser promovido. Todavia, a elevada toxicidade do Cd e a sua bioacumulação devem ser tidas em conta, tal como a vulnerabilidade dos sapais, cuja redução terá consequências drásticas para a saúde do ecossistema.

Tendo em conta a possibilidade de auto-remediação de N nos sapais através do processo de desnitrificação, o segundo caso de estudo deste capítulo, teve como objectivo testar se a desnitrificação em sapais é afectada pela contaminação por metais. Deste modo, foi comparada a taxa de desnitrificação ("^{15}N-isotope pairing technique"), durante o inverno, em sapais com diferentes níveis de contaminação por metais. As taxas de desnitrificação foram inferiores no sapal não contaminado e também inferiores em condições de luz, i.e. durante o período diurno. Nas condições testadas, a taxa diária de desnitrificação obtida foi cerca de 2285 ± 420 µmol N m^{-2} dia^{-1} no sapal de *S. maritima* não contaminado e 11046 ± 7398 µmol N

m^{-2} dia^{-1} no sapal contaminado. No entanto, a variabilidade é bastante superior no sapal contaminado. Em suma, este estudo contribuiu para avaliar a capacidade de auto-remediação dos sapais através da desnitrificação, tendo em conta stresses múltiplos, i.e. "eutrofização cultural" e "contaminação histórica" por metais. Todavia, serão úteis mais resultados comparáveis com este.

De um modo geral, a presente tese evidencia os serviços dos sapais na redução/mitigação potencial da eutrofização, ainda que, no mesmo sistema, apresente alguma variação espacial. A espécie de sapal *Spartina maritima*, nativa da Europa, contribui para a remediação de azoto através da intercepção do azoto proveniente de montante e reduzindo a descarga de azoto reactivo para o oceano. Este processo poderá ser efectuado através da incorporação de azoto na biomassa (e.g. aminoácidos e proteínas), acumulação de azoto orgânico nos sedimentos colonizados (e.g. acumulação de detritos vegetais e aumento das taxas de sedimentação) e desnitrificação. Além disso, demonstra-se a capacidade das plantas de sapal para acumular elevadas concentrações de metais e consequentemente proceder à fitoremediação do ambiente estuarino envolvente. Por último, os stresses múltiplos estudados (enriquecimento em azoto e contaminação por metais) não afectaram a capacidade de fitoremediação de Zn, Cu e Ni por *H. portulacoides*. Por outro lado, estes factores aumentaram a capacidade de acumulação de Cd por esta espécie. Todavia, a toxicidade de Cd e a bioacumulação ao longo da cadeia trófica, tal como a vulnerabilidade dos sapais não deve ser desprezada. A desnitrificação no sapal contaminado por metais (Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd e o metalóide As) foi superior na estação do ano estudada (inverno), o que sugere uma adaptação de *S. maritima* e da comunidade microbiana às elevadas concentrações de metais no sedimento. Contudo, tal como acima mencionado, será relevante obter outros resultados comparáveis. Em suma, a capacidade dos sapais de auto-remediação de azoto e metais é afectada pelos stresses múltiplos estudados. Dado que muitas funções das halófitas de sapal são específicas para cada espécie não poderemos excluir que os stresses múltiplos possam constituir uma ameaça aos serviços prestados pelos sapais e consequentemente ameaçar a saúde e o equilíbrio do ecossistema.

Palavras-chave: eutrofização; contaminação por metais; stresses múltiplos; sapais; serviços do ecossistema

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Salt marsh habitats

Salt marshes are complex ecotones, located between land and coastal water environments, naturally dynamic systems, occurring in low energy environments usually restricted to relatively sheltered areas (Best et al., 2007). Salt marshes occur mainly in five physiographic situations: in estuaries, in saline lagoons, behind barrier islands, at the heads of sea lochs, and on beach plains; and allow in all cases the accumulation of fine sediments (Best et al., 2007). Estuarine salt marshes are located in the transitional areas where rivers gradually merge into the open sea (Boorman, 2003), being influenced by the input of fresh water and also by the incoming saltwater due to the tidal activity. Thus, upper estuarine marshes have transition communities to fresh-water wetlands, which are still tidal (Boorman, 2003).

The development of salt marsh vegetation is dependent on the presence of intertidal mudflats and other supplies of sediment (Boorman, 2003) and its extent is between mid tide level and high water spring tide level (English Nature, 2004 in Best et al., 2007). Salt marsh halophytes' communities are adapted to regular immersion by the tides and spatially distributed according to the marsh's topography, the physical and chemical characteristics of the sediment and the interspecific competition conditions (Lefeuvre et al., 2003). Therefore, these ecosystems show a clear zonation according to the frequency of inundation, being the halophyte species adapted to regular immersion by the tides and highly adapted to survive in extreme conditions including submersion by the tide and wave action, high soil salinity, and smothering by deposition of sediment (Ibañez et al., 2000; Bertness and Pennings, 2000; Boorman, 2003). These halophytes may act as sediment traps, playing an important role in the settling of suspended matter (e.g. Boorman, 2003). In addition, halophytes have similar nutrient requirements to non-saline-tolerant species and like these species they need a well-developed root system for anchoring (Amos et al., 2004) and efficient uptake of nutrients (Boorman, 2003). The root-sediment interaction is complex and covers a wide range of biogeochemical processes. In non-human-impacted intertidal salt marshes, plant diversity tends to decrease from low marsh towards high marsh, while primary production follows an opposite pattern (Lefeuvre et al., 2003). Thus, salt marshes are commonly characterized by a relatively small number of highly productive marsh species (Lefeuvre et al., 2003). Indeed, these ecosystems are among the most productive in the world (e.g. McLusky and Elliott, 2004). Moreover, salt marshes are ecologically important habitats as bio-stabilisers due to the

immediate changes in the physical environment (e.g. decreasing tidal currents, wave action and sediment resuspension and enhancing sediment cohesiveness and settling of suspended matter) (Widdows and Brinsley, 2002).

Lastly, salt marshes are recognised by intergovernmental agreements (e.g. Ramsar Convention on Wetlands, http://www.ramsar.org) and/or directives (e.g. EU Habitats Directive) that provides the framework for national action and international cooperation for the conservation and wise use of wetlands, including salt marshes, and their resources. In addition, salt marshes are classified as sensitive habitat under the European Habitats Directive. Specifically, this Directive aims "*to promote the maintenance of biodiversity, taking account of economic, social, cultural and regional requirements*", and, "*whereas the preservation, protection and improvement of the quality of the environment, including the conservation of natural habitats and of wild fauna and flora, are an essential objective of general interest*" (92/43/EEC). Furthermore, the use of salt marshes to assess the ecological quality status of transitional and coastal waters has received considerable attention, especially as part of the management requisites for the implementation of the Water Framework Directive (2000/60/EC) (Best et al., 2007; Simas and Ferreira, 2007).

Ecosystem services provided by salt marshes

The ecological meaning of salt marshes was firstly recognised by Odum (1961, in Boorman, 1999) and Teal (1962, in Boorman, 1999) in the USA, and by Chapman (1960, in Boorman, 1999) and Ranwell (1972, in Boorman, 1999) in Europe. Moreover, the multiple services provided by salt marshes was firstly estimated in terms of economical value by Constanza et al. in 1997, highlighting the need to preserve these ecosystems' health.

Ecosystem functions refer to "*the habitat, biological or system properties or processes of ecosystems"*, while ecosystem services represent *"the benefits human populations derive, directly or indirectly, from ecosystem functions*" (Constanza et al., 1997). As defined by the Ecological Society of America, ecosystem services are "*the processes by which the environment produces resources that we often take for granted*" (http://www.esa.org/), or even the "*components of nature, directly enjoyed, consumed, or used to yield human wellbeing*" (Boyd and Banzhaf, 2007). Ecosystem services can be categorized in a variety of ways (NRC, 2004), and the need for several classifications was highlighted by Costanza (2008), regarding the complexity and dynamic properties of the ecosystems. The current interest in ecosystem services has come from several sources, namely the Millennium Ecosystem Assessment (MA,

2005) which highlighted the significance of ecosystem services to human wellbeing and showed that these services are threatened by unsustainable anthropogenic activities (Naidoo et al., 2008). Maintenance of hydrologic cycles, climate regulation, air and water cleansing, waste treatment, pollination, soil genesis, and storing and cycling of nutrients are some of the ecosystem services provided worldwide by salt marshes (Constanza, 1997; Daily, 1997).

Salt marsh plants' biomass and productivity, as well as role on nutrient cycling and regulation, namely denitrification, nutrient burial, climate regulation through carbon sequestration, cultural services (recreation, science and education) are important ecosystem services provided by salt marshes, through the reduction of the impact of land-derived nitrogen loads and, consequently, eutrophication (Valiela et al., 2000; Farber et al., 2006; Sousa et al., 2008b; 2010; Philipott et al., 2009). In addition, salt marsh plants are well known by their capacity to accumulate and retain metals, being able to promote the system auto-remediation through metals rhizofiltration (rhizosphere accumulation of metals through plants absorption, concentration and precipitation of contaminants from polluted aqueous sources), phytostabilization (reduction of mobility and bioavailability of metals in the soil by plant roots - complexation) or phytoaccumulation (accumulation of metals in plants biomass) (e.g. Ghosh and Singh, 2005; Sousa et al., 2008a). Furthermore, these ecosystems are also important nursery sites for juvenile fish and breeding sites for birds, stabilize shorelines and intercept land-derived nutrients (e.g. Valiela et al., 2000; McLusky and Elliott, 2004; Jin et al., 2007; Green et al., 2009). Thus, the reduction of salt marsh areas worldwide, as a result of anthropogenic disturbance is of major concern, and several studies on the ecology of estuaries have emphasized the negative consequences of its disappearance (e.g. Valiela et al., 2000; Boorman, 2003; Lefeuvre et al. 2003; Best et al., 2007; Simas and Ferreira, 2007; Wieski et al., 2010).

Multiple stressors in salt marshes: nitrogen and metals

As previously mentioned, salt marshes are characterized as a *Sensitive Habitat* under the European Habitats Directive (92/43/EEC). Boorman (2003) reviewed the factors affecting the development and sensitivity of salt marshes, dividing them into natural events and anthropogenic activities. However, this thesis concerns the anthropogenic influences, which will be from now on specified.

Global population has been increasing, and almost quadrupled since the beginning of the 20th century (Vitousek et al., 1997). Human activities have been negatively affecting the

coastal areas worldwide (Nixon, 1995), since human populations tend to mainly settle in coastal watersheds (Valiela et al., 1992; Vitousek et al., 1997; NRC, 2000; de Jonge et al., 2002), where useful goods and services are provided. Consequently, largely associated with increasing human activities and technical advancements, many changes have occurred in the environment (Vitousek et al., 1997; Galloway et al., 2008; Gruber and Galloway, 2008) affecting estuarine and salt marshes' natural cycling and dynamics. These stressors can be summarized as: coastal developments, habitat fragmentation, recreation and disturbance (range of uses of salt marshes, direct impacts of recreation, indirect impacts of human activities), introduced species (changes in the throphic structure), pollution (agricultural chemicals, industrial chemicals, oil pollution, eutrophication) and climate change, namely extreme weather events such as storm events. A *stressor* can be defined as "*a factor that extends homeostatic or protective processes beyond the limits of the normal physiological or ecological range leading to reduced fitness*" (Sibly and Calow, 1989 and Moore et al., 2002, in Segner et al., 2007). Thus, the challenge now is how the effect of a stressor is determined by the interacting effect with another stressor and how the risk of an interaction between chemical, physical and biological stressors can be assessed and predicted.

Since the mid of last century, the anthropogenic sources of metals into the aquatic systems have been reduced due to legal restrictive rules. However, metals contaminated sediments from the past, i.e, "historical contamination" are still cause for concern due to their potential release into other environmental matrices. In addition, point sources (e.g. wastewater flux and industrial runoff) and mainly non-point (diffuse) sources (e.g. agriculture runoff and atmospheric deposition in the water surface), are still responsible for the nitrogen loading to estuaries (e.g. Lillebø et al., 2007; Howarth, 2008). Thus, salt marshes contaminated with organic pollutants may also be contaminated with metals (Church et al., 2006; Quan et al., 2007; Almeida et al, 2008). Finally, the combined effect of environmental stressors may affect estuarine communities, namely salt marsh services and the ecosystem health.

References

Almeida, C.M.R., Mucha, A.P., Delgado, M.F.C., Caçador, M.I., Bordalo, A.A., Vasconcelos, M.T.S.D., 2008. Can PAHs influence Cu accumulation by salt marsh plants? Marine Environmental Research 66, 311-318.

Amos, C., Cappucci, S., Bergamasco, A., Umgiesser G., Bonardi, M., Cloutier, D., Flindt, M. R., De Nat, L. & Cristante, S., 2004. The stability of tidal flats in Venice Lagoon – the results of in situ measurements using two benthic annular clumes. Journal of Marine Systems, 51(1-4), 211-241

Bertness, M. D. and S. C. Pennings. 2000. Spatial Variation in Process and Pattern in Salt Marsh Plant Communities. Pp 39-58, In: Concepts and Controversies in Tidal Marsh Ecology, M. Weinstein (ed) Kluwer Press, Boston

Best, M., Massey, A., Prior A., 2007. Developing a saltmarsh classification tool for the European water framework directive. Marine Pollution Bulletin 55, 205–214.

Boorman, L.A., 1999. Salt marshes – present functioning and future change. Mangroves and Salt Marshes 3, 227-241.

Boorman, L.A., 2003. Saltmarsh Review. An overview of coastal saltmarshes, their dynamic and sensitivity characteristics for conservation and management. JNCC, Peterborough. On-line version at http://www.jncc.gov.uk/pdf/jncc334.pdf Accessed 14 Oct 2009

Boyd, J., Banzhaf, S., 2007. What are ecosystem services? The need for standardized environmental accounting units. Ecological Economics 63, 616-626.

Church, T.M., Sommerfield, C.K., Velinsky, D.J., Point, D., Benoit, C., Amouroux, D., Plaa, D., Donard, O.F.X., 2006. Marsh sediments as records of sedimentation, eutrophication and metal pollution in the urban Delaware Estuary. Marine Chemistry 102, 72-95.

Costanza, R., 2008. Ecosystem services: Multiple classification systems are needed. Biological Conservation 141, 350-352.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O' Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 353-360.

Daily GC (1997) *Nature's Services: Societal Dependence on Natural Ecosystems* (Island Press, Washington, DC).

de Jonge, V.N., Elliott, M., Orive, E., 2002. Causes, historical development, effects and future challenges of a common environmental problem: eutrophication. Hydrobiologia 475/476, 1-19.

Farber, S., Costanza, R., Childers, D.I., Erickson, J., Gross, K., Grove, M., Hopkinson, C.S., Kahn, J., Pincetl, S., Troy, A., Warren, P., Wilson, M., 2006. Linking Ecology and Economics for Ecosystem Management. Bioscience 56(2), 117-129.

Galloway, J.N., Townsend A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. Science 320, 889–892.

Ghosh, M., Singh, S.P., 2005. A review on phytoremediation of heavy metals and utilization of its byproducts. Applied Ecology and Environmental Research 3(1), 1-18.

Green, B. C., Smith, D. J., Earley, S. E., Hepburn, L. J., Underwood, G. J. C., 2009. Seasonal changes in community composition and trophic structure of fish populations of five salt marshes along the Essex coastline, United Kingdom. Estuarine, Coastal and Shelf Science 85 247–256.

Gruber, N., Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451, 293-296.

Howarth, R.W., 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally Harmful Algae 8, 14–20.

Ibañez, C., Curcó, A., Day Jr, J.W., Prat, N. 2000. Structure and productivity of microtidal Mediterranean coastal marshes, in: Weinstein M.P., Kreeger, D.A., (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, pp. 107-136.

Jin, B., Fu, C., Zhong, J., Li, B., Chen, J., Wu, J., 2007. Fish utilization of a salt marsh intertidal creek in the Yangtze River estuary, China. Estuarine, Coastal and Shelf Science 73, 844-852.

Lefeuvre, J-C., Laffaille, P., Feunteun, E., Bouchard, V., Radureau, A., 2003. Biodiversity in salt marshes: from patrimonial value to ecosystem functioning. The case study of the Mont-Saint-Michel bay. Comptes Rendus Biologies 326, s125-s131.

Lillebø, A. I., Teixeira, H., Pardal, M. A., Marques, J. C., 2007. Applying water quality status criteria to a temperate estuary (Mondego, Portugal) before and after the mitigation measures to reduce eutrophication symptoms. Estuarine, Coastal and Shelf Science 72, 177-187.

McLusky, D.S., Elliot, M., 2004. The Estuarine Ecosystem - Ecology, Threats, and Management, 3rd ed. Oxford University Press.

Millennium Ecosystem Assessment, 2005. *ECOSYSTEMS AND HUMAN WELL-BEING: WETLANDS AND WATER Synthesis*. World Resources Institute, Washington, DC.

Naidoo, R., Balmford, A., Costanza, R., Fisher, B., Green, R.E., Lehner, B., Malcolm, T.R., Ricketts, T.H., 2008. Global mapping of ecosystem services and conservation priorities. Proceedings of the National Academy of Sciences of the United States of America 105(28), 9495-9550.

National Research Council (NRC), 2000. Clean Coastal Waters: Understanding and reducing the effects of Nutrient Pollution, Washington, DC, 165-176.

National Research Council. 2004. *Valuing Ecosystem Services: Toward Better Environmental Decision-Making*. Washington DC: National Academy of Sciences.

Nixon, S.W., 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. Ophelia 41, 199–219.

Philipott, L., Hallin, S., Börjesson, G., Baggs, E.M., 2009. Biochemical cycling in the rhizosphere having an impact on global change. Plant and Soil 321(1-2), 61-81.

Quan, W.M., Han, J.D., Shen, A.L., Ping, X.Y., Qian, P.L., Li, C.J., Shi, L.Y., Chen, Y.Q., 2007. Uptake and distribution of N, P and heavy metals in three dominant salt marsh macrophytes from Yangtze River estuary, China. Marine Environmental Research 64, 21–37.

Segner, E., 2007. Ecotoxicology – How to assess the impact of toxicants in a Multi-Factorial Environment? In: Mothersill, C.; Mosse, I.; Seymour, C. (Eds.) Multiple Stressors: A Challenge for the Future 484pp.

Simas, T.C., Ferreira, J.G., 2007. Nutrient enrichment and the role of salt marshes in the Tagus estuary (Portugal). Estuarine, Coastal and Shelf Science 75, 393-407.

Sousa, A.I., Caçador, I., Lillebø, A.I., Pardal, M.A., 2008a. Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites. Chemosphere 70, 850–857.

Sousa, A.I., Lillebø, A.I., Caçador, I., Pardal, M.A., 2008b. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628–635.

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I., 2010. Productivity and nutrient cycling in salt marshes: Contribution to ecosystem health. Estuarine, Coastal and Shelf Science 87, 640-646.

Valiela, I., Cole, M.L., Mcclelland, J., Hauxwell, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes, in: Weinstein, M.P., Kreeger, D.A., (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, 23-38.

Valiela, I., Foreman, K., Lamontagne, M., Hersh, D., Costa, J., Peckol, P., Brawley, J., Lajtha, K., 1992. Coupling of watersheds and coastal waters: sources and consequences of nutrient enrichment in Waquoit Bay, MA. Estuaries 15, 443– 457.

Vitousek, P.M., Aber, J., Bayley, S.E, Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, G.D., 1997. Human alteration of the global nitrogen cycle: Causes and consequences. Ecological Applications 7, 737-750.

Widdows, J., Brinsley, M., 2002. Impact of biotic and abiotic processes on sediment dynamics and the consequences to the structure and functioning of the intertidal zone. Journal of Sea Research 48, 143-156.

Wieski, K., Guo, H., Craft, C.B., Pennings, S.C., 2010. Ecosystem Functions of Tidal Fresh, Brackish, and Salt Marshes on the Georgia Coast. Estuaries and Coasts 33(1), 161-169.

GENERAL AIM

Estuaries and salt marshes are among the most productive ecosystems in the world. In addition, salt marshes are widely known to provide crucial ecosystem services, namely nutrient cycling and phytoremediation, contributing to maintain the ecosystem health. However, due to the global increasing population, particularly in the last century, salt marshes have been subdued to many anthropogenic pressures, such cultural eutrophication and historical contamination. Therefore, both eutrophication and high metals accumulation in estuarine sediments may co-occur, constituing multiple stressors.

Nitrogen is usually the liming nutrient in estuarine systems, constituting the increase of land-driven nitrogen loading the major cause of concern. Salt marshes can store nitrogen in plants biomass and detritus, which may re-enter the biogeochemical cycle, as inorganic N forms, via decomposition. However, these processes may be very much related to plants life cycles. Furthermore, nitrification-denitrification coupled process promotes the removal of the excess of nitrogen from the system to the atmosphere (the major reservoir of nitrogen). Denitrification can be seen as a process that permits maintaining the ecosystem health counteracting eutrophication.

Lastly, it has been shown that the ability of salt marsh plants to transport oxygen to the belowground parts, where it is used for root respiration and oxidation of the rhizosphere, can stimulate metals accretion and decrease their availability.

However, salt marsh functions are complex and several and open questions may arise, namely:

- Is the nitrogen cycling in salt marsh plants and their nitrogen sequestration capacity species-specific?
- What are the major factors affecting nitrogen sequestration capacity in different salt marshes colonised by the same species?
- How significant are denitrification processes in salt marshes?
- How do salt marsh plants cope with high metals contamination?
- Which are salt marshes responses to multiple stressors resulting from anthropogenic pressures?
- How can nitrogen availability affect the capacity of salt marshes for metals retention?
- How can metals affect denitrification processes at the salt marshes rhizosphere?

Thus, the present thesis focuses on the effect of multiple stressors on auto-remediation capacity of estuarine systems and its consequences to the ecosystem services. These questions will be addressed in the Chapters I, II and III of this thesis.

THESIS OUTLINE

Chapter I addresses the nitrogen cycling in salt marshes through three case studies aiming to assess: i) the contribution of different salt marsh halophytes (*Spartina maritima, Scirpus maritimus, Halimione portulacoides, Sarcocornia fruticosa, Sarcocornia perennis*) to nutrient cycling and sequestration in warm-temperate salt marshes; ii) the role of *Spartina maritima* in nitrogen retention capacity and cycling by comparing two warm-temperate systems, the Mondego and Tagus estuaries; iii) how significant are potential nitrification and denitrification processes in salt marshes, i.e., the contribution of salt marshes to N_2 removal comparatively to sediments without vegetation.

Chapter II addresses metals (Zn, Pb, Co, Cd, Ni and Cu) contamination in salt marshes, intending to understand the *Halimione portulacoides* (L.) Aellen strategies to prevent metal toxicity, showing the metal location in different plant organs and in the cell. A sequential extraction was done on leaves, stems and roots of *H. portulacoides* in order to determine and compare the metal concentration in several fractions (ethanolic, aqueous, proteic, pectic, polissacaridic, lenhinic and cellulosic) of the plant material.

CHAPTER III addressess the influence of multiple stressors on the auto-remediation processes occurring in salt marshes. In order to better understand how is the salt marsh plants auto-remediation capacity (phytoaccumulation of metals) affected by cultural eutrophication, an experiment was performed under controlled conditions. *Halimione portulacoides* plants were exposed to equal metal concentrations (Zn, Cu, and Ni – micronutrients, and Cd – class B metal) simulating historical contamination and three different concentrations of nitrogen (nitrate) simulating steps of cultural eutrophication. In addition, denitrification in *Spartina maritima* salt marshes from two warm-temperate estuaries with different historical metal contamination were compared. It was hypothesized that denitrification, as a service provided by *S. maritima* marshes, may be affected by the presence of metals, namely Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and the metalloid As.

CHAPTER I

N Cycling In Salt Marshes

CHAPTER I - N cycling in salt marshes

Nitrogen can be found in sedimentary and crystalline rocks but the major fraction of nitrogen at Earth's surface is in the atmosphere (e.g. Tolstikhin and Marty, 1998). In addition, nitrogen is an essential element of all biological life, since it is associated to proteins pool (organic nitrogen), being often the limiting nutrient for net primary production (NPP) in estuarine and marine systems (Matamala and Drake, 1999). The nitrogen cycle between the environmental compartments and the biota is quite complex and involves several processes mediated by microorganisms, i.e., nitrogen (N_2 gas) fixation, nitrogen incorporation of inorganic forms (NO_3^-, NH_4^+) and transformation into organic forms (e.g. aminoacids, proteins), nitrogen mineralization, nitrification and denitrification (NO_3^- conversion to N_2 gas). So, in non-human-impacted environments, reactive nitrogen results from biological nitrogen fixation. In addition, between nitrogen fixation and denitrification, reactive nitrogen is utilized by ecosystems and distributed among earth's reservoirs (i.e. atmosphere, ocean, terrestrial and marine biota and soil/sediment organic matter) (Galloway, 1998). According to Galloway (1998) review, and considering non-human-impacted environments, most of the land-derived nitrogen loads to coastal environments could be denitrified in estuarine and shelf regions. Therefore, under these circumstances, the cycle should be approximately in balance with little accumulation of reactive nitrogen in coastal areas. However, the supply of reactive nitrogen to global terrestrial ecosystems has increased as a consequence of world population and human activity (e.g. Galloway, 1998; Galloway et al., 2008). The changing of the nitrogen cycle as a result of the production and industrial use of artificial nitrogen fertilizers worldwide has induced environmental problems, such as eutrophication of terrestrial and aquatic systems (e.g. Hauxwell and Valiela, 2004; Gruber and Galloway, 2008).

Concerning the management of coastal systems, namely transitional waters, there is an increasing need to remedy long-lasting adverse effects of human interventions and much effort has been put into reducing eutrophication. Towards this goal, conservation and restoration of coastal areas, has become a priority during the last decades (e.g. Valiela et al., 2000; McLusky and Elliott, 2004; Lillebø et al., 2007). Once in the aquatic system land-derived nitrogen loads may be trapped in biota or in the sediment, or passed to its main compartment, the atmosphere, reducing the availability of reactive nitrogen. Although there are still uncertainties concerning the fate of all land-derived nitrogen (Galloway et al., 2004), denitrification seems to be an important reactive nitrogen sink, meaning that even in heavily altered regions, rivers, although important sources of nitrogen to coastal systems, represent

small sources of reactive nitrogen to the open ocean (Galloway et al., 2008). Thus, primary production, organic nitrogen burial and denitrification constitute processes that may trap reactive nitrogen in estuaries (Nedwell et al., 1999; Valiela, 2000; Sousa et al., 2008, 2010). Hence, considering the role of primary producers in this process, salt marshes may have a crucial part in nitrogen balance. Thus, the central question of this chapter is to address the salt marshes' meaning on nitrogen remediation, by intercepting the land-derived nitrogen and buffering the loading of reactive nitrogen to the open ocean.

Eutrophication in coastal ecosystems

Over the 20th century, eutrophication in coastal ecosystems spread all over the world as a result of the increase in nutrient loading (*e.g.* Nixon, 1995; Vitousek et al., 1997; Bricker et al., 1999; de Jonge et al., 2002; Hauxwell and Valiela, 2004; Lillebø et al., 2007; Kemp et al. 2005; Fisher et al., 2006; McKlathery et al., 2007). Eutrophication can be defined as "*an increase in the rate of supply of organic matter to an ecosystem*" (Nixon, 1995). This widely accepted definition focuses on the supply of carbon and energy to an ecosystem, distinguishing the phenomenon from its causes and consequences (Nixon, 2009). Later, eutrophication was also defined as ''*the enrichment of water by nutrients, especially compounds of nitrogen and/or phosphorus causing an accelerate growth of algae and higher forms of plant life to produce and undesirable disturbance to the balance of organisms present in the water and to the quality of the water concerned*'' (UWWT Directive 91/271/EEC) (Crouzet et al., 1999).

Major natural supplies of nutrients to estuarine water column include: 1) external sources, like diffusive runoff from agriculture lands, freshwater discharge from rivers or anthropogenic point sources (urban or industrial discharges, sewage treatment works); and 2) endogenous processes, such as salt marsh production, benthic sediment mineralization and sediment interstitial waters. Thus, eutrophication can be a consequence of human induced nutrient loadings (point or diffuse) (*cultural eutrophication*) and also of the system endogenous processes (namely increased mineralization of organic matter due to tidal action) (de Jonge et al., 2002). In estuarine regions where human population is dense, the nutrient supply is mainly increased due to anthropogenic loading such as domestic and industrial waste waters, urban drainage and agricultural effluents; meaning that nutrient loadings are somehow related to the global population development (*e.g.* de Jonge et al., 2002; Tappin, 2002; Hauxwell and Valiela, 2004).

Eutrophication is mainly driven by nitrogen loadings, even though phosphorus loadings can also lead/contribute to coastal eutrophication (NRC, 2000; Howarth and Marino, 2006). Nitrogen pollution sources due to human activity include synthetic nitrogen fertilizers, agricultural sources due to biological nitrogen fixation and creation of reactive nitrogen through fossils fuels burning (Galloway et al., 2004; Howarth et al., 2008). Eutrophication represents the worldwide main agent of change for coastal ecosystems (Crouzet et al., 1999) and a wide range of responses to eutrophication can occur/have been observed. The most direct is the hypoxia and anoxia due to decomposition of organic matter, a shift in primary producers from vascular plants to opportunistic algae, a reduction of seagrass beds, increase in phytoplankton biomass and increase of water turbidity; thus, water quality changes in freshwater and marine ecosystems (Valiela et al., 1997; Raffaelli et al, 1998; Howarth et al., 2000; NRC, 2000; de Jonge et al., 2002; Smith, 2003). The occurrence of hypoxia and anoxia, and consequently the shift of primary producers, depend on the hydrology of the system and can lead several consequences such as habitat degradation, poor water quality, loss of biodiversity, alteration of food-web structure (including the fish community composition) and increase harmful algal blooms (de Jonge et al., 2002; NRC, 2000; Lillebø et al., 2005; Kemp et al., 2005).

Nitrogen in salt marshes

Nitrogen (N) is most often the limiting nutrient of primary production in coastal marine ecosystems, even though phosphorus can also be limiting on a seasonal or regional basis (Fisher et al., 1999). In salt marshes, N can be found both in inorganic and organic forms, as well as in different oxidation states (it undergoes several oxidation/reduction reactions). Microbial communities, specially autotrophic and heterotrophic bacteria, play a key role on these reactions being strongly dependent on the physico-chemical conditions (Herbert et al., 1999; Purvaja et al., 2008). Even though 78% of the atmosphere is composed by dinitrogen gas (N_2) (being the largest reservoir of N), N_2 is somehow considered biologically unavailable since it cannot be directly uptake by most primary producers.

The N cycle can be divided in several steps:

- *N_2 fixation*, transporting N to the bioavailable pool, is performed by benthic cyanobacteria (autotrophic or heterotrophic prokaryotes), which convert dinitrogen gas into ammonium (NH_4^+). This step of the N cycle occurs in the euphotic zone.

- *Ammonification/mineralization* step is performed by heterotrophic bacteria and fungi and constitutes the mineralization of organic N to NH_4^+. This process can be aerobic or anaerobic.
- *Ammonia volatilization* is a physicochemical process where ammonia in the ammonium-ammonia equilibrium is transformed into the gaseous form and released to the atmosphere.
- *Nitrogen assimilation* is the conversion of inorganic N to organic N in plant cells and tissue, contributing to the removal of N. Plants' uptake of inorganic N can have different meaning depending on the season.
- *Nitrification* is the obligate aerobic oxidation of NH_4^+ to nitrate (NO_3^-) by nitrifying bacteria, which occurs through two-step reactions: oxidation of NH_4^+ to NO_2^- and NO_2^- oxidation into NO_3^-. This process is then coupled to a low oxygen/anaerobic process (denitrification).
- *Denitrification* consists in the NO_3^- loss to the atmosphere as nitrous oxide (N_2O) and/or dinitrogen (N^2).
- *Anaerobic ammonium oxidation* (*anammox*) is a process mediated by oxidizing bacteria which convert ammonium to nitrite which in turn is converted to nitrate.

Anammox contribution to N removal from the water column has different meanings, depending on the water depth. It seems to be higher at deep waters rather than on shallow waters (Dalsgaard et al., 2005). Denitrification is the dominant process of nitrate reduction in most shallow marine sediments (Herbert, 1999). However, *dissimilatory nitrate reduction of ammonium* (*DNRA*), the alternative pathway of nitrate reduction, may also be important under certain conditions.

As stated by Gruber and Galloway (2008) "*There is compelling evidence that human alteration of the nitrogen cycle is negatively affecting human and ecosystem health*". As a result of energy and food production, the nitrogen oxides and ammonia emitted are efficiently spread in the atmosphere and deposited on the terrestrial and aquatic systems in a form that is readily available to primary producers, thereby stimulating productivity (Philipott et al., 2009). As an outcome of increasing global population and increasing human activities coastal systems, namely transitional waters, became subjected to increasing of land-derived nitrogen loads, often leading to eutrophication (e.g. Hauxwell and Valiela, 2004; Lillebø et al., 2007; Howarth, 2008). In fact, eutrophication has been classified as a worldwide agent of change for

coastal ecosystems (NRC, 2000). Therefore, nutrient cycling in coastal ecosystems is a crucial function performed by salt marshes (Nixon, 1981), acting as transformers of nutrients.

Denitrification in salt marshes

Denitrification, the stepwise reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to the gaseous nitric oxide (NO), nitrous oxide (N_2O) and, mainly, dinitrogen (N_2), is a process mediated by anaerobic bacteria. Under suboxic conditions, these bacteria can use NO_3^- (or NO_2^-) as a terminal electron acceptor in respiration. Denitrification process occurs in the sediment suboxic surface and includes denitrification of the NO_x (NO_3^-+ NO_2^-) of bottom water and of NO_x produced via sedimentary nitrification (this step is called coupled nitrification-denitrification).

Nitrification (the microbial aerobic oxidation of NH_4^+ and NO_2^- to NO_3^-) is an important step of the nitrogen cycle that occurs in the oxic surface sediment. The product of this process (NO_3^-) may be later on denitrified (coupled nitrification-denitrification). The potential nitrification is affected by environmental factors like temperature, oxygen and NH_4^+ availability, but also by the microbiological community, i.e, number and activity of nitrifying bacteria (Henrikson et al., 1981), and it is an essential step for the coupled nitrification-denitrification (Koop-Jacobsen and Giblin, 2009).

Salt marshes have been recognized as important players on the nitrogen cycle by enhancing the removal of the excess of reactive nitrogen through denitrification (e.g. Teal and Howes, 2000; Valiela and Cole, 2002; Galloway et al., 2008). This process, by counteracting eutrophication in coastal areas (Seitzinger, 1988), constitutes an ecosystem service provided by salt marshes (Costanza, 1997). Moreover, denitrification is considered the only significant biochemical pathway, mediated by denitrifying bacteria, that regenerates N_2 (Schlesinger, 1997; Jaffe, 2000). This process enables the reduction of the loading of reactive nitrogen to the open ocean, up to 40-50 % of the inorganic nitrogen (Seitzinger, 1988), meaning that rivers become small sources of reactive nitrogen to the open ocean (Galloway et al., 2008).

Several studies have been carried out in order to quantify denitrification in freshwater wetlands (e.g. Merrill and Cornwell, 2000; Risgaard-Petersen, 2003; Trimmer et al., 2003; Sundbäck et al., 2006) but much less studies have addressed salt marshes (e.g. Valiela and Teal 1979; Koch et al., 1992; White and Howes, 1994; Erickson et al., 2003; Poulin et al., 2007).

References

Bricker SB, Clement CG, Pirhalla DE, Orlando SP, Farrow DRG (1999) National Estuarine Eutrophication Assessment: effects of nutrient enrichment in the nation's estuaries. NOAA, National Ocean Service, Centers for Coastal Ocean Science, Silver Spring, MD

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O' Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 353-360.

Crouzet, P., Leonard, J., Nixon, S., Rees, Y., Parr, W., Laffon, L.,Bøgestrand, J., Kristensen, P., Lallana, C., Izzo, G., Bokn, T., Back, J.,Lack, T.J., 1999. Nutrients in European ecosystems. In: Thyssen, N.(Ed.), Environmental Assessment Report n_4. European EnvironmentalAgency, p. 82. http://reports.eea.eu.int/.

Dalsgaard, T., Thamdrup, B., Canfield, D.E. 2005. Anaerobic ammonium oxidation (anammox) in the marine environment, Research in Microbiology 156, 457-464.

de Jonge, V.N., Elliott, M., Orive, E., 2002. Causes, historical development, effects and future challenges of a common environmental problem: eutrophication. Hydrobiologia 475/476, 1-19.

Eriksson, P.G., Svensson, J.M., Carrer, G.M., 2003. Temporal changes and spatial variation of soil oxygen consumption, nitrification and denitrification rates in a tidal salt marsh of the Lagoon of Venice, Italy. Estuarine, Coastal and Shelf Science 58, 861-871.

Fisher, T.R., Gustafson, A.B., Sellner, K., Lacouture, R., Haas, L.W., Wetzel,R.L., Magnien, R., Everitt D., Michaels, B., Karrh, R., 1999. Spatial and temporal variation of resource limitation in Chesapeake Bay. Marine Biology 133, 763-778.

Fisher, T.R., Hagy III, J.D., Boynton, W.R., Williams, M.R., 2006. Cultural eutrophication in the Choptank and Patuxent estuaries of Chesapeake Bay. Limnology and Oceanography 51(1-2), 435-447.

Galloway, J.N. Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions. Science 320, 889-892.

Galloway, J.N., 1998. The global nitrogen cycle: changes and consequences. Environmental Pollution 102, 15-24.

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P. Asner, G.P, Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M. Michael, A.F., Porter, J.H., Townsend, A.R., Vörösmarty, C.J., 2004. Nitrogen cycles: past, present, and future. Biogeochemistry 70: 152-226.

Gruber, N., Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451, 293-296.

Hauxwell, J., Valiela, I., 2004. Effects of nutrient loading on shallow seagrass-dominated coastal systems: patterns and processes. In Nielsen, G. Banta, and M. Pedersen (eds) Estuarine Nutrient Cycling: The influence of Primary Producers. Kluwer Academic Publishers, London, p 59-92.

Henriksen, K., Hansen, J.I., Blackburn, T.H., 1981. Rates of nitrification, distribution of nitrifying bacteria, and nitrate fluxes in different types of sediment from Danish waters. Marine Biology 61, 299-304.

Herbert, R.A., 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews 23, 563-590.

Howarth, R., 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmfull Algae 8, 14-20.

Howarth, R., 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmfull Algae 8, 14-20.

Howarth, R.W., Anderson, D., Cloern, J., Elfring, C., Hopkinson, C., Lapointe, B., Malone, T., Marcus, N., McGlathery, K., Sharpley, A., Walker, D., 2000. Nutrient pollution of coastal rivers, bays, and seas. Issues Ecol. 7, 1–15.

Howarth, R.W., Marino, R.M., 2006. Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over 3 decades. Limnol. Oceanogr. 51, 364–376.

Jaffe, D.A., 2000. The nitrogen cycle, p. 322–342. *In* M. C. Jacobson, R. J. Charlson, H. Rodhe, and G. H. Orians (ed), Earth system science. Academic Press, San Diego, Calif.

Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde, E. D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M., Stevenson, J.C., 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. Marine Ecology Progress Series 303, 1–29.

Koch, M.S., Malby, E., Oliver, G.A., Bakker, S.A., 1992. Factor controlling denitrification rates of tidal mudflats and fringing salt marshes in south-west England. Estuarine, Coastal and Shelf Science 34, 471–485.

Koop-Jakobsen, K., Giblin, A.E., 2009. Anammox in Tidal Marsh Sediments: The Role of Salinity, Nitrogen Loading, and Marsh Vegetation. Estuaries and Coasts 32, 238–245.

Lillebø, A.I., Teixeira, H., Pardal, M.A., Marques, J.C., 2007. Applying quality status criteria to a temperate estuary before and after the mitigation measures to reduce eutrophication symptoms. Estuarine, Coastal and Shelf Sciences 72, 177-187.

Matamala, R. Drake, B.G., 1999. The influence of atmospheric CO2 enrichment on plant-soil nitrogen interactions in a wetland plant community on the Chesapeake Bay. Plant and Soil 210, 93-101.

McGlathery, KJ., Sundbäck, K., Anderson, I.C., 2004. The importance of primary producers for benthic nitrogen and phosphorus cycling, in: Nielsen, S., Banta, G., Pedersen, M., (Eds.), Estuarine nutrient cycling: The influence of primary producers. Kluwer Academic Publishers, The Netherlands, pp. 231-261.

McLusky, D.S., Elliot, M., 2004. The Estuarine Ecosystem - Ecology, Threats, and Management, 3rd ed. Oxford University Press Merrill and Cornwell, 2000;

National Research Council (NRC), 2000. Clean Coastal Waters: Understanding and reducing the effects of Nutrient Pollution, Washington, DC, 165-176.

Nedwell, D.B., Jickells, T.D., Timmer, M., Sanders, R., 1999. Nutrients in estuaries, in: Nedwell, D.B., Raffaelli, D.G., (Eds), Estuaries. Advances in Ecological Research 29, 43-92.

Nixon, S.W., 1981. Remineralization and nutrient cycling in coastal marine ecosystems. In: Neilson, B.J., Cronin, L. E. (eds) Estuaries and nutrients. Humana Press. Clifton. NJ. p 111-138

Nixon, S.W., 1995. Coastal marine eutrophication: a definition, social causes, and future concerns. Ophelia 41, 199–219.

Nixon, S.W., 2009. Eutrophication and the macroscope. Hydrobiologia 629, 5-19.

Philipott, L., Hallin, S., Börjesson, G., Baggs, E.M., 2009. Biochemical cycling in the rhizosphere having an impact on global change. Plant and Soil 321(1-2), 61-81.

Poulin, P., Pelletier, E., Saint-Louis, R., 2007. Seasonal variability of denitrification efficiency in northern salt marshes: An example from the St. Lawrence Estuary. Marine Environmental Research 63, 490–505.

Purvaja, R., Ramesh, R., Ray, A.K.,Rixen, T., 2008. Nitrogen cycling: A review of the processes, transformations and fluxes in coastal ecosystems. Indian Agriculture, Environment and Health. Current Science, 94 (11), 1419-1438.

Raffaelli, D.G., Raven, J. and L. Poole, 1998. Ecological impact of green macroalgal blooms. Annual Review of Marine Biology and Oceanography 36, *97*-125.

Risgaard-Petersen, N., 2003. Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediment: on the influence of benthic microalgae. Limnology and Oceanography 48, 93–105.

Schlesinger, W.H., 1997. Biogeochemistry, 2nd ed. Academic Press, San Diego, Calif.

Seitzinger, S.P., 1988. Denitrification in fresh and coastal marine ecosystems: ecological and geochemical significance. Limnology and Oceanography 33, 702–724.

Smith, V.H., 2003. Eutrophication of freshwater and coastal marine ecosystems: A global problem. Environmental Science and Pollution Research 10, 1–14.

Sousa, A.I., Lillebø, A.I., Caçador, I., Pardal, M.A., 2008. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628–635.

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I., 2010. Productivity and nutrient cycling in salt marshes: Contribution to ecosystem health. Estuarine, Coastal and Shelf Science 87, 640-646.

Sundbäck, K., Miles, A., Linares, F., 2006. Nitrogen dynamics in nontidal littoral sediments: Role of microphytobenthos and denitrification. Estuaries and Coasts 29(6), 1196-1211.

Tappin, A.D., Harris, J.R.W., Uncles, R.J., Boorman, D., 2002. Potential modification of the fluxes of nitrogen from the Humber Estuary catchment (U.K.) to the North Sea in response to changing agricultural inputs and climate patterns. Hydrobiologia 475/476, 65–77.

Teal, J.M., Howes, B.L., 2000. Salt marsh values: retrospection from the end of the century. In: Weinstein, M. P. and D. A. Kreeger. Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishing. Dordrecht, the Netherlands, p 9-22.

Tolstikhin, I. N. & Marty, B., 1998. The evolution of terrestrial volatiles: a view from helium, neon, argon and nitrogen isotope modelling. Chemical Geology, 147, 27-52.

Trimmer, M., Gowen, R.J., Stewart, B.M., 2003. Changes in sediment processes across the western Irish Sea front. Estuarine, Coastal and Shelf Science 56, 1011-1019.

Valiela I, Collins G, Kremer J, Lajtha K, Geist M, Seely B, Brawley J, Sham CH. 1997a. Nitrogen loading from coastal watersheds to receiving estuaries: new method and application. Ecol Appl 7:358–80.

Valiela, I., Cole, M.L., 2002. Comparative evidence that salt marshes and mangroves may protect seagrass meadows from land-derived nitrogen loads. Ecosystems 5, 92–102.

Valiela, I., Cole, M.L., Mcclelland, J., Hauxwell, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes, in: Weinstein, M.P., Kreeger, D.A., (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, 23-38.

Valiela, I., Teal, J.M., 1979. The nitrogen budget of a salt marsh ecosystem. Nature 280, 652-656.

White, D.S., Howes, B.L., 1994. Long-term ^{15}N-nitrogen retention in the vegetated sediments of a New England salt marsh. Limnology and Oceanography 39(8), 1878-1892.

Case studies

1. Productivity and nitrogen cycling in salt marshes: contribution to ecosystem health

Abstract

This study aimed to assess the contribution of different salt marsh halophytes (*Spartina maritima*, *Scirpus maritimus*, *Halimione portulacoides, Sarcocornia fruticosa, Sarcocornia perennis*) to nutrient cycling and sequestration in warm-temperate salt marshes. Nitrogen concentration in plant organs and rhizosediment, as well as plant biomass were monitored every two months during one year. Results show that halophytes colonizing the upper and middle marsh areas had the highest NBPP (net belowground primary production) as well as the retention of nitrogen (N) in the rhizosediment. Yet, excluding *S. maritimus*, all halophytes seem to contribute to the retention of N from external sources. It seems that there is no relation between plants sequestration capacity for nitrogen and plant photosynthetic pathway. This work shows that nutrient cycling and accumulation processes by salt marsh halophytes contribute to reduce eutrophication (N retention), highlighting salt marsh ecosystems services and the crucial role of halophytes in maintaining ecosystem functions and health.

Keywords: Nutrient cycling, Halophytes, Nitrogen

Introduction

In warm temperate estuaries, salt marshes are often colonized by the halophytes *Spartina maritima*, *Scirpus maritimus*, *Halimione portulacoides*, *Sarcocornia fruticosa*, and *Sarcocornia perennis*. Although different halophytes are adapted to different physico-chemical characteristics, colonizing upper, middle or lower marshes, they may all have an important role, contributing to the ecosystem high productivity. In addition, despite differences in their physiology and annual biological cycle, these halophytes may function as nutrient buffers namely for nitrogen (Sousa et al., 2008); wherein the biomass production contributes to the removal of nutrients from the system, and their cycling in estuarine systems (Ibañez et al., 1999). These ecosystem processes represent salt marsh services, which should gain greater importance considering main threats to these coastal areas, i.e. the reduction of salt marsh areas worldwide, as a result of anthropogenic disturbance (e.g. Best et al., 2007); the increasing nutrient loadings with anthropogenic origin, named as “cultural” eutrophication;

and the observed global warming (increase [CO_2] and temperature). Moreover, the way higher CO_2 levels will affect the primary productivity is not well known (Ericksson et al., 2007; Kakani et al., 2008). Understanding the primary productivity of salt marshes requires the knowledge of photosynthetic pathways of salt marsh halophytes, which is one of the adaptations to these environmental conditions. The C_3 and C_4 photosynthetic pathways are morphologically and physiologically different and adapted to specific environmental conditions (Table 1), which, in part, determines the geographical location of different plant species. The C_4 photosynthetic pathway is a specialized adaptation from the C_3 ancestor's at the metabolic and morphologic level (Kranz anatomy), which allows increased performance of C_4 plants when the CO_2 availability is low and the temperature is warm (Ehleringer et al., 1997).

Table 1. Plant species and their physico-chemical adaptations to C_3 and C_4 photosynthetic pathway.

	Photosynthetic pathway	
	C_3	**C_4**
Plant species	*Halimione portulacoides* *Scirpus maritimus* *Sarcocornia fruticosa* *Sarcocornia perennis*	*Spartina maritima*
Temperature and CO_2	Adapted to low temperatures [(a)] and high CO_2 [(b)]	Adapted to warm temperature and low CO_2 conditions [(a,d)]
Light	-	High light conditions [(c)]
Morphological and metabolic advantage	-	Kranz anatomy: specialized adaptation from C_3 ancestors [(d)]
Ability to acclimate	High ability to acclimate to low light, temperature variation or elevated CO_2 [(c)]	Low ability to acclimate to adapted/changed environments [(c)] At low salinity: higher photosynthetic capacity than C_3 [(e)]
Other aspects	-	Higher potential productivity than C_3 plants [(f)]

(a) Kakani et al., 2008
(b) Ehlringer, 1993
(c) Sage and McKown, 2006
(d) Ehleringer et al., 1991, 1997
(e) Nieva et al., 1999
(f) Adam, 1990

S. maritima is distributed along the coasts of western, southern and southeastern Europe, and western Africa. It is an herbaceous perennial plant, with a C_4 type of photosynthetic mechanism (Nieva et al., 1999; Gray and Mogg, 2001) and colonizes low marshes. *S. maritimus* L., is widely distributed in Europe (Peláez et al., 1998) and North America (Kantrud, 1996) and it usually forms dense monospecific stands in shallow brackish marshes. This species has a C_3 type of photosynthetic mechanism (Boschker et al., 1999). *H. portulacoides* (L.) Aellen is pointed out as one of the most abundant halophytes in European salt marshes (Bouchard et al., 1998), colonizing low and mid-marsh areas. It is a small Chenopodiaceae shrub species and has a C_3 photosynthetic mechanism. *S. fruticosa* L. (Scott), is a succulent chenopod shrub and usually colonizes mid to high marshes (Redondo-Gómez et al., 2006). It is found along the Mediterranean coasts and South Africa and has a C_3 photosynthetic pathway (Abdulrahman and Williams, 1981). *S. perennis* (Mill.) A. J. Scott, is distributed along the western and southern coasts of Europe and the southern coasts of Africa. It is a halophytic subshrub with succulent and articulated stems, with a C_3 photosynthetic pathway (Davy et al., 2006), typically found on low to intermediate levels of salt marshes.

Do different plant species have a different ability to mitigate eutrophication?

The aim of this work was to test if productivity (biomass) and nitrogen cycling in different halophyte species (common in warm-temperate estuarine systems), with different photosynthetic pathways, had the same ability to reduce eutrophication. This case study will allow for an estimation of the contribution of different salt marsh plants towards mitigating eutrophication.

Materials and methods

Five halophyte species (*S. fruticosa*, *S. perennis, H. portulacoides, S. maritimus and S. maritima*) were sampled every two months for one year (from June to April). Sampling took place in monospecific and homogeneous stands at warm temperate salt marshes located in the southern European Atlantic margin (Portugal). *S. fruticosa*, *S. perennis, H. portulacoides* and *S. maritima* were sampled in the Tagus estuary (site 1, 38°44'37.11''N, 9°03'43.49''W); and *S. maritimus* and *S. maritima* were sampled in the Mondego estuary (site 2, 40°07'11.87''N, 8°50'25.96''W), located two hundred kilometres north of site 1. *S. maritima* was collected at low level of the salt marsh and all the other studied species were collected at intermediate level of salt marshes. The aboveground material was sampled by collecting 0.3x0.3 m^2 squares (three replicates) and the belowground material was collected through sediment cores (Ø 7 cm

and 25 cm depth). In the laboratory, the collected plant material was separated into different parts (leaves, stems and belowground material) and rinsed with demineralised water. Then, it was dried until constant weight at 60°C. Extra rhizosediment samples (sediment among plant roots and rhizomes (Almeida et al., 2006)) were also collected to determine the total content nitrogen. These samples were air dried, separated from roots and passed through a 0.25 mm mesh.

Previous to analysis, all plant material and sediment were ground and homogenised. Total nitrogen concentrations were quantified in a CHNS/O analyser (Fisons Instruments Model EA 1108).

Aboveground and belowground biomass productions were calculated by subtracting the minimum biomass (an average of all replicates per date) from the maximum biomass (also an average of all replicates per date) (De la Cruz and Hackney, 1977). Maximum and minimum biomass values were obtained considering all the sampling dates. Nitrogen pools were calculated by multiplying the biomass per the N concentration in the plants tissues. In turn, the standing stocks in the plant material were calculated by subtracting the minimum pools from the maximum values. Considering the NBPP (as N standing stocks) and subtracting it from the N stored in the sediment we obtain the N inputs to the sediment that can be explained by coming from external sources.

Results

All sediments consisted mainly of fine particles between 63 µm and 125 µm (silt and clay, data not shown). The five studied halophytes are perennial and the aboveground and belowground biomass productions are shown in Figure 1.

Results show that the majority of the halophytes have a greater contribution (> 50 %) of belowground material to the total biomass production, than aboveground material to the total. However, *S. fruticosa* and *S. maritima* from site 2 have 59 % of aboveground biomass production. *S. fruticosa* had the highest total biomass production, being about 4 times higher than *S. perennis. S. maritima* from both sites showed similar belowground biomass production, yet in site 2 the aboveground production is three times higher. *S. maritimus* showed the smallest proportion of aboveground production, corresponding to 15 % of total biomass production. *H. portulacoides* and *S. perennis* had similar total biomass production, as well as the proportions of the above and belowground biomass productions.

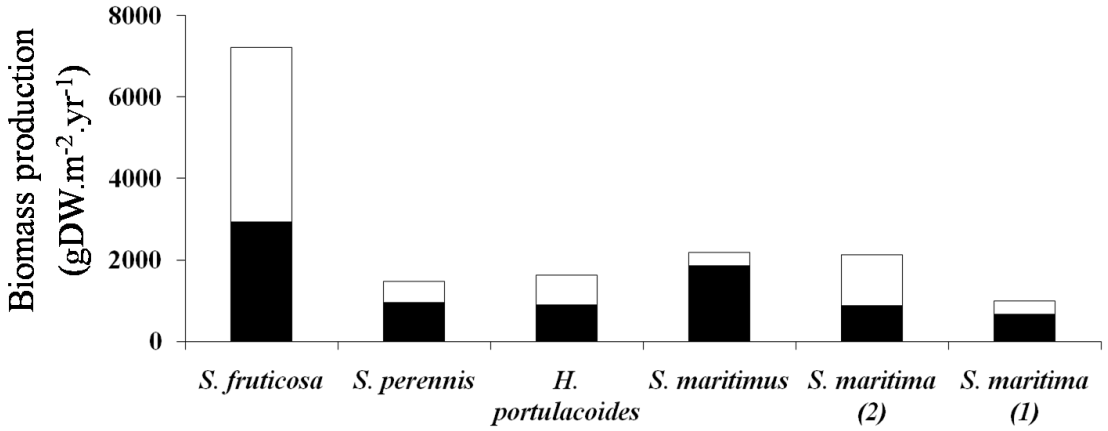


Figure 1. Biomass production for aboveground (white column) and belowground (black column) material, from June to April. Photosynthetic pathway (C_3 and C_4) is indicated for each species and *S. maritima* sampling site (1) and (2) is indicated in the x axis.

Nitrogen cycling in each of the studied salt marshes is shown in Figure 2. Results show that halophytes colonizing the middle marsh areas (*S. fruticosa, S. perennis* and *H. portulacoides*) have the highest NBPP as well as the retention of N in the rhizosediment (57 to 59 g N m^{-2} y^{-1}). Moreover, 37 to 50 % of the N retained in the rhizosediment comes from external sources. *S. maritima* colonizing lower marsh areas of both systems had a similar N retention in the rhizosediment (24 and 29 g N m^{-2} y^{-1}). However, in *S. maritima* (1) rhizosediment 64 % of the N retained comes from external sources, whilst in *S. maritima* (2) the NBPP contributes with 98 % of the N retained in the rhizosediment. *S. maritimus*, colonizing the middle marsh areas, showed a comparatively lower N retention capacity in the rhizosediment (21 g N m^{-2} y^{-1}) and 14% of the NBPP is washed out from the rhizosediment. N use efficiency (the amount of N used to produce a certain amount of plant biomass) varied with plant species, and *S. maritima* (1) is the most efficient using N in the aboveground biomass production (NAPP). However, *S. maritima* (2) only has a better N use efficiency than *S. maritimus*. Regarding N use efficiency in the belowground material, *S. maritima* (1) and *S. maritima* (2) are most efficient than *S. perennis* and *H. portulacoides*, but less efficient than *S. fruticosa* and *S. maritimus*. Looking at N use efficiency in total biomass production, *S. maritima* (1) only has a lower efficiency than *S. fruticosa*, but *S. maritima* (2) is more efficient in the use of N than *S. perennis* and *H. portulacoides*, but lower efficient than *S. fruticosa*, *S. maritima* (1) and *S. maritimus*.

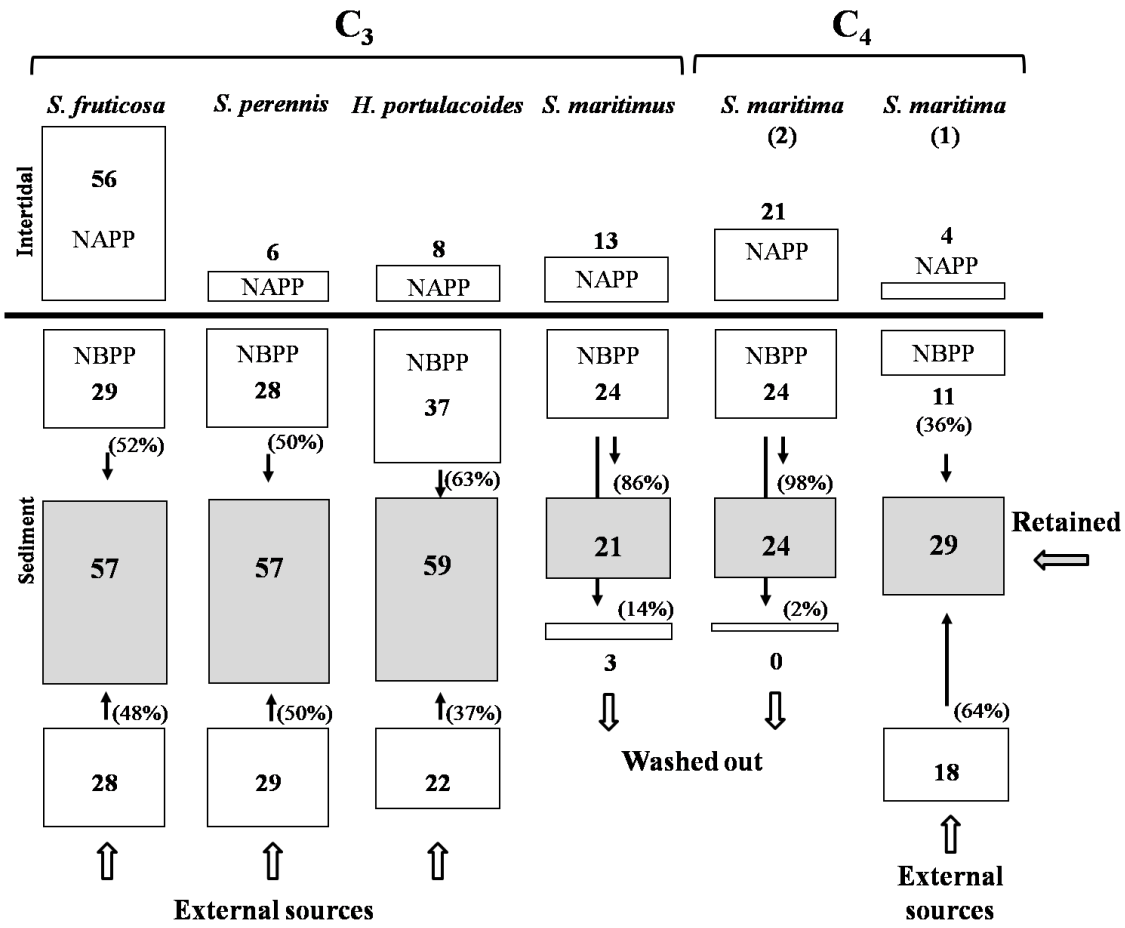


Figure 2. Nitrogen cycling in salt marshes colonized by 5 halophyte species. NAPP (net aboveground primary production)/standing stock in the aboveground, NBPP (net belowground primary production)/standing stock in the belowground, and the nitrogen sequestration in the sediment (due to the belowground plant production and some external sources) are shown in the scheme. The numbers in bold refer to $gN.m^{-2}.y^{-1}$

Discussion

S. maritima biomass production (both above and belowground) depends on the physico-chemical characteristics (Ibañez et al., 1999; Curcó et al., 2002) and the maturity of the salt marshes (Sousa et al., 2008, 2010). In salt marshes, the development of belowground biomass is enhanced by halophytes under environmental stress (e.g. low nutrient availability, tidal inundation, high soil salinity) (Ibañez et al., 1999; Scarton et al., 2002; Sousa et al., 2008). In the present study, *S. maritima* from both sites showed similar belowground biomass production, yet in site 2 the aboveground production was three times higher. *S. maritima* (2) above: belowground biomass production ratio (≈ 0.5) was found to have a similar value to values found for the same species in created marshes in Spain (≈ 0.6) (Castillo et al., 2008).

This low ratio was also reported in *Spartina alterniflora* (Windham et al., 2003). In *S. fruticosa* above: belowground biomass production ratio was 1.47 (59% of aboveground and 41% of belowground biomass production), which is a much higher value than those found by other studies in temperate salt marshes. For instance, in the Po Delta (Italy), *S. fruticosa* above: belowground biomass production ratio was 0.54 (Scarton et al., 2002) and is 0.61 at Ebre Delta (Spain) (Curcó et al., 2002). Higher belowground than aboveground biomass production, as stated above, may be a response to stressing environmental conditions, such as flooding and high interstitial salinity (e.g. Scarton et al., 2002). This suggests that the studied *S. fruticosa* is not stressed by nutrient limitation, which allows it to invest in aboveground biomass production. In contrast, the aboveground production of *S. maritimus* corresponded to 15% of total biomass production. Unlike the other studied halophytes, which have a slow continuous growth and do not show a seasonal dieback, *S. maritimus* photosynthetic shoots are active for a single growing season reaching the mature phase in the spring, while the standing dead biomass follows in the summer and fall periods and only belowground parts persist into the next year (Lillebø et al., 2003). This distinct behavior may explain the differences observed in N cycling in the salt marsh colonized by *S. maritimus*.

Nitrogen content in plant tissue is directly related to the N availability in each ecosystem and strongly influences the net primary production, since N is usually a limiting nutrient in these systems (Matamala and Drake, 1999). Thus, above and belowground biomass and nitrogen standing stocks reflect N content and nutrient availability to each studied plant. Adding to the NBPP contribution to N enrichment of the sediment, most of the halophytes' sediments are N enriched by external sources, increasing the amount of N retained in the sediment. *S. fruticosa*, *S. perennis* and *H. portulacoides* contributed most to N sequestration in the studied salt marshes. Regarding N use efficiency, even though several studies have shown that C_4 plants are more efficient than C_3 (e.g. Jocic and Saric, 1983; Sage and Pearcy, 1987; Sage et al., 1987), this work shows that *S. maritima* (C_4) is not always more efficient than the studied C_3 species. Moreover, *S. maritima* from different locations (sites 1 and 2) showed different N use efficiency, corroborating a previous study (Sousa et al., 2008). Thus, it is not the photosynthetic pathway alone that determines the N use efficiency of these plants as other parameters may be involved in this process. In this study, the predicted economy in N use by C_4 plants is not seen in the N dynamics.

Nitrogen dynamics is influenced by salt marsh plants (Lillebø et al., 2006) meaning that different halophytes contributed differently to N cycling within marshes. For instance, *S. maritimus* NBPP contributed with the highest percentages N washed out from the rhizosediment, i.e. 14 % N. In addition, *S. maritimus* was the only halophyte that did not retain

N from external sources in the rhizosediment. These results are probably related to the high biomass turnover rate of this halophyte above and belowground material (respectively 0.92 and 0.42 y^{-1}). Salt marsh plants themselves contribute to retain and accumulate N in their biomass (Sekiranda and Kiwanuka, 2004; Sollie and Veroheven, 2008; Sousa et al., 2008). Thus, constructed wetlands have been developed as an efficient removal process of N from the system. For instance, *Phragmites australis* (in the Mar Menor), which is adapted to nutrients' healthy ecosystems, was proposed as a way to reduce eutrophication in this lagoon by accumulating nutrients in its biomass (Ruiz and Velasco, 2009).

These halophytes play an important role N cycling and sequestration that varies with the halophyte. *S. fruticosa* and *S. perennis* as well as *H. portulacoides* seemed to be the most efficient for N sequestration. Since these halophytes have different photosynthetic pathways (i.e. CAM – *S. fruticosa* and *S. perennis*, C_3 – *H. portulacoides* and *S. maritimus*, C_4 – *S. maritima*) and different efficiency in nitrogen retention, no clear relationship between the photosynthetic pathway and the sequestration efficiency could be found.

Conclusions

Salt marsh plants provide an important service through the production of biomass. They contribute to a greater stability and lower erosion of the coastal areas and increase pollution retention (e.g. Castillo et al., 2008). Nitrogen uptake from sediment interstitial waters and their incorporation in plant biomass imply nutrient sequestration and retention, thus decreasing its availability in the water column and potentially reducing eutrophication. According to our results, there is no strict and consistent relation between biomass production, nitrogen cycling in salt marshes and each salt marsh plant species' photosynthetic pathway.

Global climate change will affect salt marshes and estuaries in terms of photosynthesis, growth, biomass allocation and nutrient uptake by plants (Gavito et al., 2001; Kakani et al., 2008) and consequently salt marsh services are likely to be affected. Future studies should be performed in order to better understand these halophyte's ability to acclimate to new environmental conditions (increasing atmospheric CO_2 and temperature), and to monitor how the ecosystem services and health are affected.

References

Abdulrahman, F.S., Williams, G.J., 1981. Temperature and salinity regulation of growth and gas exchange of *Salicornia fruticosa* (L.) L. Oecologia (Berlin) 48, 346-352.

Adam, P., 1990. Plants and Salinity in Salt Marsh Ecology. Cambridge University Press, Cambridge, pp. 208-277.

Almeida, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D, 2006. Variability of metal contents in the sea rush *Juncus maritimus*–estuarine sediment system through one year of plant's life. Marine Environmental Research 61, 424–438.

Best, M., Massey, A., Prior A., 2007. Developing a saltmarsh classification tool for the European water framework directive. Marine Pollution Bulletin 55, 205–214.

Boschker, H.T.S., de Brouwer, J.F.C., Cappenberg, T.E., 1999. The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediment: Stable isotop analysis of microbial biomarkers. Limnology and Oceanography 44, 309-319.

Castillo, J.M., Leira-Doce, P., Rubio-Casal, A.E., Figueroa, E., 2008. Spatial and temporal variations in aboveground and belowground biomass of *Spartina maritima* (small cordgrass) in created and natural marshes. Estuarine Coastal and Shelf Science 78, 819–826.

Curcó, A., Ibañez, C., Day, J.W., Prat, N., 2002. Net primary production and decomposition of salt marshes of the Ebre Delta (Catalonia, Spain). Estuaries 25, 309-324.

Davy, A.J., Bishop, G.F., Mossman, H., Redondo-Gómez, S., Castillo, J.M., Castellanos, E.M., Luque, T., Figueroa, M.E., 2006. Biological Flora of the British Isles: *Sarcocornia perennis* (Miller) A.J. Scott. Journal of Ecology 94, 1035–1048.

De la Cruz, A.A., Hackney, C.T., 1977. Energy value, elemental composition, and productivity of belowground biomass of a *Juncus* tidal marsh. Ecology 58, 1165-1170.

Ehleringer, J.R., Monson, R.K., 1993. Evolutionary and ecological aspects of photosynthetic pathway variation. Annual Review of Ecology and Systematics 24, 411-439.

Ehleringer, J.R., Sage, R.F., Flanagan, L.B., Pearcy, R.W., 1991. Climate change and the evolution of C4 photosynthesis. Trends in Ecology and Evolution 6, 95-99.

Ehleringer, J.R., Thure, E., Cerling, B., Helliker, R., 1997. C_4 photosynthesis, atmospheric CO_2, and climate. Oecologia 112, 285-299.

Erickson, J.E., Megonical, J.P., Peresta, G, Drake, B.G., 2007. Salinity and sea level mediate elevated CO_2 effects on C_3–C_4 plant interactions and tissue nitrogen in a Chesapeake Bay tidal wetland. Global Change Biology 13, 202–215.

Gavito, M.E., Curtis, P.S., Mikkelsen, T.N., Jakobsen, I., 2001. Interactive effects of soil temperature, atmospheric carbon dioxide and soil N on root development, biomass and nutrient uptake of winter wheat during vegetative growth. Journal of Experimental Botany 52, 1913-1923.

Gray, A.J., Mogg, R.J., 2001. Climate impacts on pioneer saltmarsh plants. Climate research 18, 105–112.

Ibañez, C., Day, Jr.J.W., Pont, D. 1999. Primary production and decomposition of wetlands of the Rhône Delta, France: interactive impacts of human modifications and relative sea level rise. Journal of Coastal Research 15, 717–731.

Jocic, B., Saric, M.R., 1983. Efficiency of nitrogen, phosphorus, and potassium use by corn, sunflower, and sugarbeet for the synthesis of organic matter. Plant and Soil 72, 219-223.

Kakani, V.G., Surabhi, G.K., Reddy, K.R., 2008. Photosynthesis responses of C_4 plant *Andropogon gerardii* acclimated to temperature and carbon dioxide. Photosynthetica 46, 420-430.

Kantrud, H.A., 1996. The alkali (*Scirpus maritimus* L.) and saltmarsh (*S. robustus* Pursh) bulrushes: A literature review. National Biological Service, Information and Technology Report 6. Jamestown ND: Northern Prairie Wildlife Research Center Online. http://www.npwrc.usgs.gov/resource/plants/bulrush/index.htm Assessed 24 October 2009.

Lillebø, A.I., Flindt, M.R., Pardal, M.A., Marques, J.C., 2006. The effect of *Zostera noltii, Spartina maritima* and *Scirpus maritimus* on sediment pore-water profiles, in a temperate intertidal estuary. Hydrobiologia 555, 175-183.

Lillebø, A.I., Pardal, M.A., Neto, J.M., Marques, J.C., 2003. Salinity as the major factor affecting *Scirpus maritimus* annual dynamics. Evidence from field data and greenhouse experiment. Aquatic Botany 77, 111-120.

Matamala, R., Drake, B.G., 1999. The influence of atmospheric CO_2 enrichment on plant-soil nitrogen interactions in a wetland plant community on the Chesapeake Bay. Plant and Soil 210, 93–101.

Nieva, F.J.J., Castellanos, E.M., Figueroa, M.E., Gil, F., 1999. Gas exchange and chlorophyll fluorescence of C3 and C4 saltmarsh species. Photosynthetica 36, 397-406.

Peláez, F., Collado, J., Arenal, F., Basilio, A., Cabello, A., Díez Matas, M.T., García, J.B., González del Vale, A., González, V., Gorrochategui, J., Hernández, P., Martin, I., Platas, G., Vicente, F., 1998. Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. Mycological Research 102, 755-761.

Ruiz, M., Velasco, J., 2010. Nutrient bioaccumulation in *Phragmites australis*: management tool for reduction of pollution in the Mar Menor. Water, Air and Soil Pollution 205, 173-185.

Sage, R.F., McKown, A.D., 2006. Is C4 photosynthesis less phenotypically plastic than C3 photosynthesis? Journal Experimental Botany 57, 303-317.

Sage, R.F., Pearcy, R.W., 1987. The nitrogen use efficiency of C3 and C4 plants. II. Leaf nitrogen effects on the gas exchange characteristics of *Chenopodium album* (L.) and *Amaranthus retroflexus* (L.). Plant Physiology 84, 959-963.

Sage, R.F., Pearcy, R.W., Seemann, J.R., 1987. The Nitrogen Use Efficiency of C3 and C4 Plants. III. Leaf nitrogen effects on the activity of carboxylating enzymes in *Chenopodium album* (L.) and *Amaranthus retroflexus* (L.). Plant Physiology 85, 355-359.

Scarton, F., Day, J.W., Rismondo, A., 2002. Primary Production and Decomposition of *Sarcocornia fruticosa* (L.) Scott and *Phragmites australis* Trin. Ex Steudel in the Po Delta, Italy. Estuaries 25, 325-336.

Sekiranda, S.B.K., Kiwanuka, S., 1998. A study of nutrient removal efficiency of *Phragmites mauritianus* in experimental reactors in Uganda. Hydrobiologia 364, 83–91.

Sollie, S., Verhoeven, J.T.A., 2008. Nutrient Cycling and Retention Along a Littoral Gradient in a Dutch Shallow Lake in Relation to Water Level Regime. Water, Air and Soil Pollution 193, 107-121.

Sousa, A.I., Lillebø, A.I., Caçador, I., Pardal, M.A., 2008. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628-635.

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I. 2010. The influence of *Spartina maritima* on carbon retention capacity in salt marshes from warm-temperate estuaries. Marine Pollution Bulletin 61, 215-223.

Windham, L, Weis, J.S., Weis, P., 2003. Uptake and distribution of metals in two dominant salt marsh macrophytes, *Spartina alterniflora* (cordgrass) and *Phragmites australis* (common reed). Estuarine, Coastal and Shelf Science 56, 63–72.

2. Contribution of salt marshes to the reduction of eutrophication in estuarine systems

Abstract

Salt marshes are among the most productive ecosystems on the world, performing important ecosystem functions, namely nutrient recycling. In this study, the role of *Spartina maritima* in nitrogen retention capacity and cycling on Mondego and Tagus estuaries is compared. Two mono-specific *Spartina maritima* stands per estuary were studied during one year, concerning biomass, nitrogen pools, litter production and decomposition rates. Results showed that the oldest Tagus salt marsh population presented higher net annual belowground biomass and nitrogen productions, as well as the slower decomposition rate for litter, contributing to the higher N accumulation in the sediment; whereas *Spartina* marshes in Mondego estuary presented higher values of aboveground production. Detritus moved by tides represented a huge amount of total aboveground production, probably significant when considering the N balance of these salt marshes. Results reinforce salt marshes functions namely by trapping nitrogen and consequently contribute to reduction of eutrophication in transitional waters.

Keywords: *Spartina maritima*, nitrogen, salt marsh, bioremediation, eutrophication

Introduction

Salt marshes, estuaries and other coastal waters have been subdue to increasing nitrogen loadings with anthropogenic origin, as a result of increasing global population and increasing human activities (e.g. Vitousek et al., 1997; see Herbert et al., 1999). These high increases in nitrogen (N) loadings often conduct many estuaries to eutrophication (Valiela and Bowen, 2002; Lillebø et al., 2005), thus representing the worldwide main agent of change for coastal ecosystems (NRC, 2000). Since the productivity of coastal ecosystems is highly determined by N, salt marshes receiving high N loads exhibit an increase in primary production (see Herbert et al., 1999; McLusky and Elliott, 2004).

Salt marshes are among the most productive ecosystems in the world (e.g. McLusky and Elliott, 2004), and the high value of the multiple services provided by wetlands, including salt marshes, has already been estimated (Constanza et al., 1997), highlighting the need to preserve these ecosystems' health. The productivity of salt marshes is affected by several

inter-related parameters, such as tidal regime (flooding frequency and duration), soil salinity, temperature, rainfall, nutrient availability, oxygen levels, sediment type, etc. (Ibañez et al., 2000). Moreover, the ecological significance of salt marshes, namely as nursery areas for fishes and breeding sites for birds (McLusky and Elliott, 2004) is largely recognized. They have also an important role as bio-stabilisers, preserving their immediate physical environment by decreasing tidal currents, wave action and sediment resuspension and enhancing sediment cohesiveness and settling of suspended matter (Widdows and Brinsley, 2002). Nutrient cycling in the coastal ecosystems is another crucial role performed by salt marshes (Nixon, 1980), acting as transformers of nutrients (either functioning as sinks and/or sources) (Ibañez et al., 2000). The decision of functioning as sinks and/or sources, depends on several factors such as successional age of the tidal marsh, tidal energy, salinity, estuarine sources, assimilatory nutrient uptake, N fixation, leakage by live and grazed autotrophs, oxygen release, nutrient production and losses due mineralization and nitrification-denitrification, etc. (Ibañez et al., 2000; Eyre and Ferguson, 2002; McGlathery et al., 2004). Estuaries can be sediment traps and consequently, as already mentioned, can act as a sink for nutrients, which reach these systems as particulate nutrients through river fluxes (Jickells et al., 2005). Primary production, organic N burial and denitrification within the sediment constitute processes, which may trap the N in the estuaries (Nedwell et al., 1999). Hence, considering the plants role on this process, salt marshes have a crucial role on N remediation.

Salt marsh plants possess a well-developed aerenchyma system, which confers them the ability to transport oxygen to the belowground parts (Maricle and Lee, 2002), where it is used for root respiration and oxidation of the rhizosphere. Therefore, these plants may interfere with benthic nutrient cycles, also by modification of redox potentials and pH within the sediment, which lastly influence processes such as nutrient adsorption to particles, and several steps of the N cycle (ammonification, nitrification, denitrification and N-fixation) (see Pedersen et al., 2004).

According to the “Outwelling Hypothesis”, due to tidal inundation, part of the marsh-estuarine primary productivity is exported to the coastal ocean supporting near coastal ocean productivity (Nixon, 1980; Odum et al., 1995). Thus, litter accumulation on these marshes depends on tides intensity, flooding frequency, and also on the wind. Additionally, decomposition rate of plant material in salt marshes depends on tidal inundation (Bouchard and Lefeuvre, 2000), which in turn influences the sediment environmental conditions (Foote and Reynolds, 1997).

Spartina maritima (Curtis) Fernald is a rhizomatous grass with a continuous but very slow growth forming extensive monotypic stands, and occupies intertidal mudflats both at

Mondego and Tagus (Portugal) estuaries. Depending on *Spartina* morphology and stage of vegetation development in the system, they can act as a sink or source of nitrogen (e.g. Odum et al., 1995). This study intends to reveal the role of *S. maritima* on N cycling on salt marshes at these two warm-temperate mesotidal estuaries. Which is the *S. maritima* contribution to the reduction of eutrophication in estuarine and coastal areas by removing excess N from these systems?

Materials and methods

Study sites

S. maritima was studied at two warm-temperate mesotidal estuaries (Mondego and Tagus) located on the Atlantic coast of Portugal (40°08' N, 8°50' W and 38°49' N, 08°56' W, respectively).

The Mondego estuary is about 7 km long and is 2-3 km across at its widest part and drains a hydrological basin of 6670 km^2. It comprises two different arms, northern and southern, separated by an alluvium-formed island (Murraceira island) (Figure 1a). The northern arm is deeper (4-8 m during high tide, tidal range 1-3 m) and constitutes the principal navigation channel and the location of Figueira da Foz harbour. The southern arm is shallower (2-4 m during high tide, tidal range 1-3 m), and is characterised by large areas of intertidal flats exposed during low tide, wherein *S. maritima* salt marshes colonise 4% of the lower estuarine areas (Coelho et al., 2004). Two different salt marshes in the south arm were selected: Gala, in the outer part of the estuary (and consequently more influenced by the tidal circulation) and Jusante (Esteiro dos Armazéns), located in an inner part (Figure 1a).

The Tagus estuary constitutes one of the largest estuaries in the western coast of Europe. It has a shallow bay covering an area of about 320 km^2 and the river drains an area of 86 000 km^2 (Figure 1b). Seawater enters the estuary through a deep narrow inlet channel and tides are semi-diurnal with average amplitude of 2.4 m ranging from 0.9 m at neap tide to 4.1 m at spring tide (Gameiro et al., 2004). This study was carried out at Pancas and Corroios salt marshes. Pancas is located in the eastern bank of the estuary, is a younger and bigger (800 ha) salt marsh with extensive intertidal mudflats, and is included in a Nature Reserve (RNET). In turn, Corroios is located in the southern suburbia of Lisbon, is an older/mature and smaller salt marsh (400 ha) and receives effluent discharges mainly from urban and industrial sources, considering the proximity of urbanized and industrial areas. Both marshes are colonized by *S. maritima* (Caçador et al., 2004).

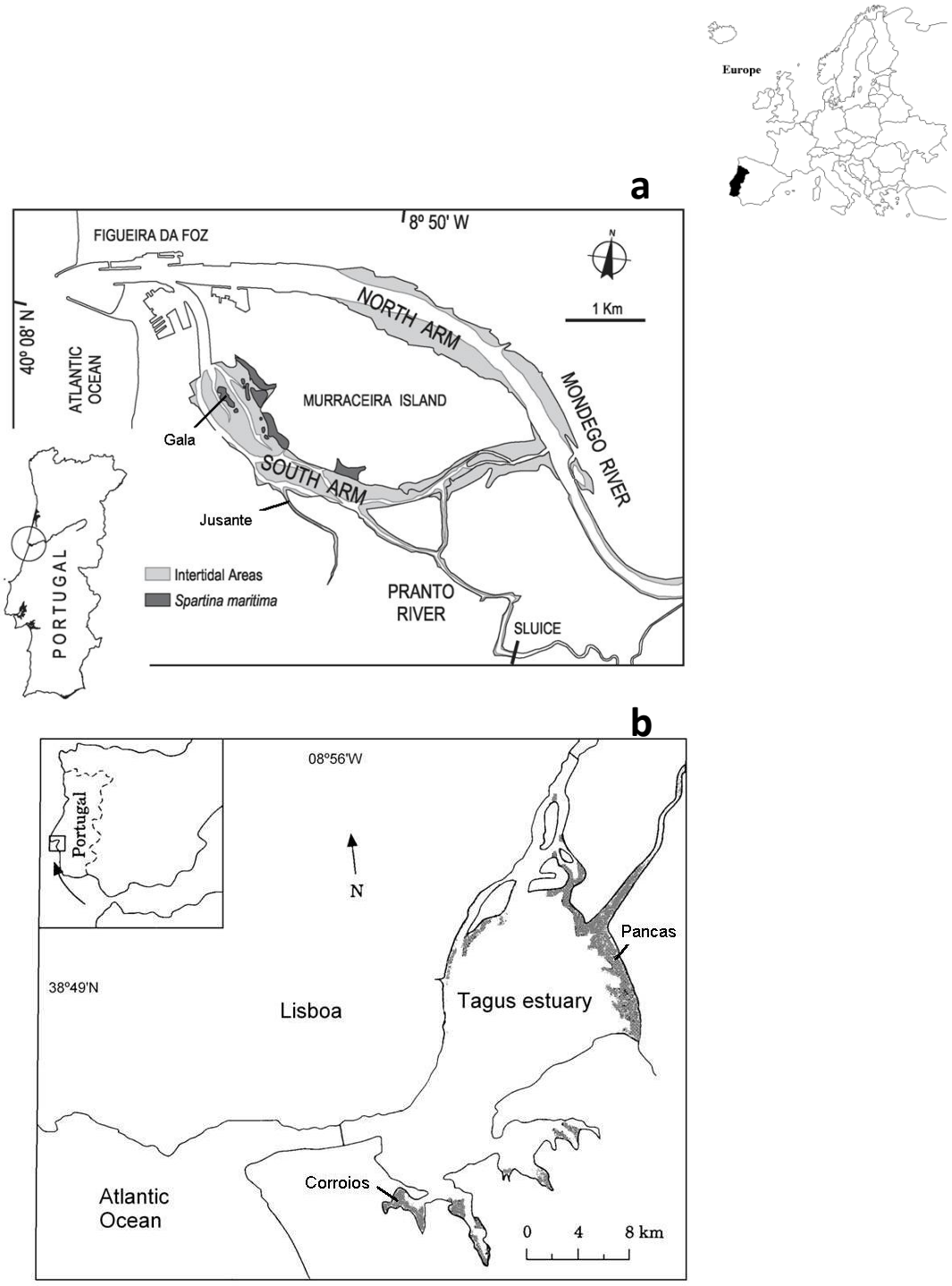


Figure 1 - Sampling sites at the Mondego (a) and Tagus (b) estuaries.

Sampling strategy

S. maritima samples were collected bi-monthly for a one-year period at the Tagus salt marshes. The sampled stands were uniform in size and in density of stems. At the Mondego salt marshes, five random samples were collected and aboveground material was clipped at ground level for a circle of Ø 7 cm. Detritus production at Mondego salt marshes was

estimated from Castro (1999). At the Tagus salt marshes, it was sampled three squares of 0.3x0.3 m^2 and the detritus accumulated on the square sediment was removed by hand and transported to the laboratory in plastic bags. After this procedure, sediment cores were taken exactly on the same sites, in order to collect the correspondent belowground material. It was used a circular core of Ø 7 cm and 25 cm depth at the Mondego and Tagus *Spartina* stands. Other rooted sediment samples were monthly collected to determine the total N and organic matter content. Subsequently, all the samples were transported to the laboratory to be processed.

***S. maritima* processing**

At the laboratory, the collected aboveground and belowground material were rinsed with demineralised water, aboveground part separated into stems and leaves and all plant dried until constant weight (48 h) at 60 ^{0}C. This procedure was also performed with the detritus collected on the sediment surface.

Biomass and nitrogen production

Biomass production (aboveground and belowground) was estimated according to the differences between the maximum and minimum biomass recorded during all the year studied, as described by De la Cruz and Hackney (1977) for belowground material. Nitrogen production was estimated in the same way considering maximum and minimum nitrogen pool values. Nitrogen pool was calculated by multiplying the biomass per the nitrogen concentration of *S. maritima*. Turnover rate for aboveground and belowground biomass and nitrogen was also calculated (the ratio between biomass production and the maximum biomass, and the ratio between nitrogen production and the maximum nitrogen pool, respectively).

Litterbag field experiment

In order to analyze the decomposition of belowground vegetation, belowground components were collected from several random locations at Gala and Jusante marshes in June and at Pancas and Corroios marshes in February. The samples were rinsed and dried and then, 10x10 cm^2 nylon mesh bags with 450 μm diameter holes containing about 5 g of belowground material were placed in the field. Each bag was individually weighted. In order to reproduce, as closely as possible, their natural habitat, the bags were buried at 10 cm depth in their own environments. Five bags (replicates) at Mondego and three bags at Tagus were collected periodically between June and December, and between February and September at Mondego and Tagus marshes, respectively. The remaining belowground biomass data were

fitted to an exponential decay model (first order decay function), $X_t=X_0*e^{-kt}$, where X_t is final/remaining dry weight in litterbags, X_0 is initial dry weight, t is time in days and k is the decay constant/rate (see Curcó et al., 2002).

Analytical procedures

Plant material from the Mondego salt marshes was dried to constant weight at 105 ^{0}C and analyzed for total nitrogen content (CHN-analyzer, Carlo Erba). Mondego sediment samples were grounded and homogenized, and total nitrogen was analyzed according to standard methods described in Limnologisk Metodik (1992). Sediment samples from the Tagus salt marshes were air dried, cleaned of roots with tweezers and passed through a 0.25 mm mesh. For N quantification, sediments and biological material were also grounded and homogenized.

Total N concentration was quantified in sediment samples, above and belowground biomass, detritus and litterbag material (these also at the beginning time/day of the experiment) using a CHNS/O analyser (Fisons Instruments Model EA 1108). Sediment triplicate sub-samples were analyzed for loss of ignition, after 8h at 450 ^{0}C.

Results

Sediment characterization

Some physico-chemical parameters of the salt marsh sediments are described in Table 1 and in Figure 2.

Table 1. Sediment granulometry for Mondego and Tagus salt marshes.

	Granulometry
Gala	silt and clay: 6% fine sand: 56% (63µm-125µm) 38% (>125µm)
Jusante	silt and clay: 15% fine sand: 54% (63µm-125µm) 31% (>125µm)
Pancas	silt: 60% clay: 38% [(a)]
Corroios	

[(a)] Caçador et al. (2004).

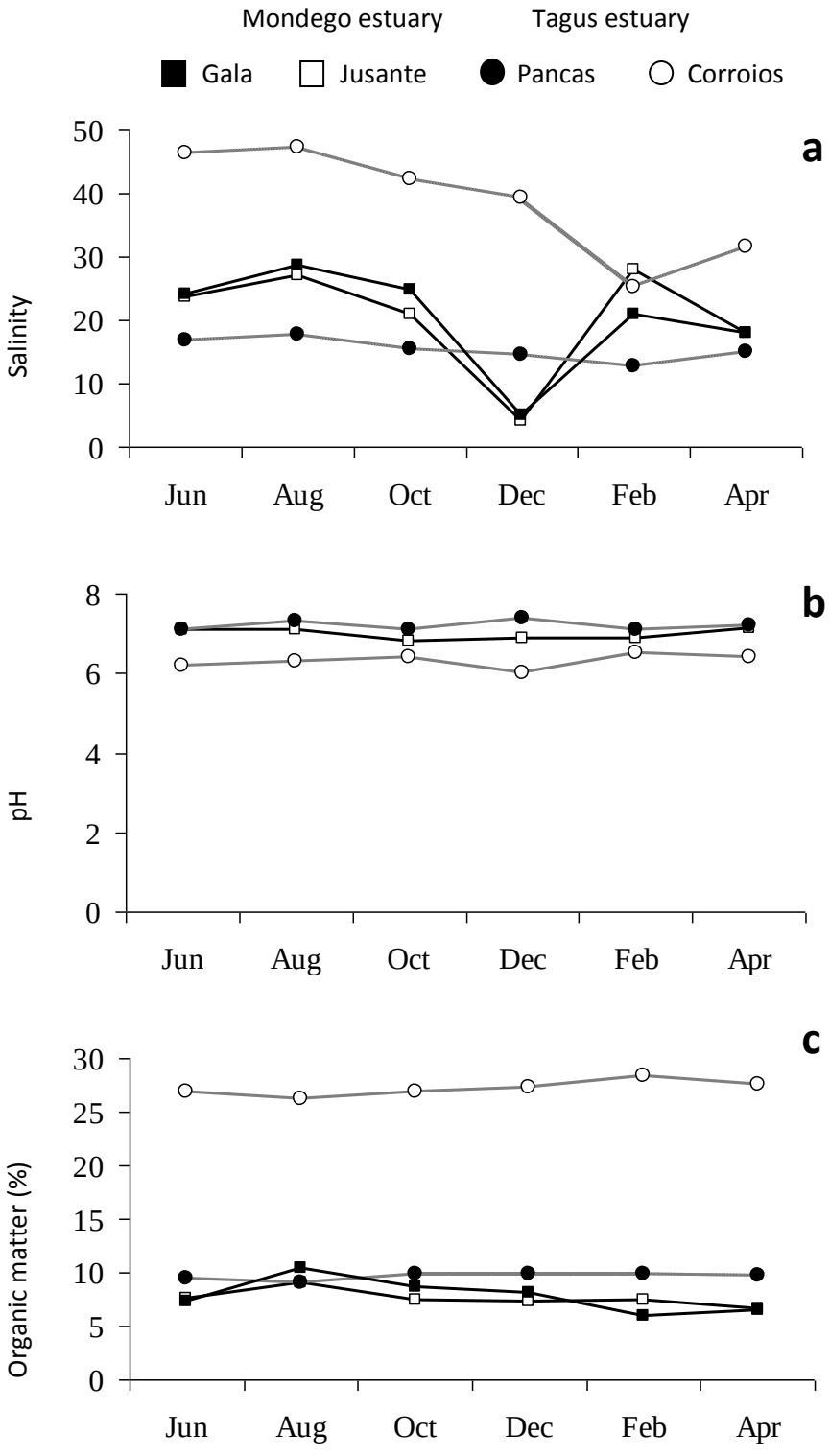


Figure 2. *Spartina* rooted sediment characteristics in the salt marshes of the Mondego and Tagus estuaries; (a) salinity, (b) pH and (c) organic matter content.

Concerning salinity, Pancas presented slightly lower values ranging from 13 to 19; Gala and Jusante presented similar values, ranging from 18 to 29 with an exception in December, due to a raining period. Corroios was the saltier salt marsh presenting values from 23 to 47 (Figure 2a). The pH was similar during all the year, ranging between 6 and 7.4, wherein Corroios showed the lowest pH values, meaning higher plant effect at the rhizosphere (Figure 2b). Considering organic matter content, both salt marshes from Mondego estuary present about 8% and Pancas about 9%, whereas Corroios present a quite higher O.M. content of 27% (Figure 2c). Tagus salt marshes present a huge percentage of silt and clay, thus retaining mainly fine particles. Mondego salt marshes present coarser sediments than Tagus, even if it is fine sand (Table 1).

Above- and belowground biomass

Gala and Jusante presented higher aboveground (leaves and stems) biomass of *S. maritima* comparing to belowground biomass, whereas Pancas and Corroios presented the opposite situation, with belowground biomass being considerably higher than aboveground biomass (Table 2). The highest aboveground biomass values were registered at Gala, followed by Jusante, Pancas and lastly Corroios, with the lowest aboveground biomass values. On the contrary, Corroios presented the highest belowground biomass values, decreasing to Jusante, and with lower values occurring at Gala and Pancas. In the Mondego salt marshes there was not a clear seasonal pattern in *S. maritima* biomass (Table 2). At Pancas it can be observed a seasonal variation in *Spartina* above and belowground biomass, with maximum values occurring in August/October and a minimum in February. Corroios did not present a clear seasonal variation in *Spartina* aboveground biomass, but belowground biomass increased from October to June.

Nitrogen pool

Considering both the biomass ($gDW.m^{-2}$) and nitrogen concentration (%) of *S. maritima* and multiplying them, the nitrogen pool ($gN.m^{-2}$) at leaves, stems and belowground parts was calculated and is shown in Figure 3. According to the biomass patterns, nitrogen pool was higher in the aboveground part of *Spartina* at Gala, while at Pancas and Corroios it was the belowground part that presented the highest N pool. Jusante presented higher nitrogen pool in belowground than aboveground material.

Table 2 . *Spartina maritima* leaves, stems and belowground biomass in Mondego and Tagus salt marshes for a one year period (average ± SD; n=5 (Mondego); n=3 (Tagus) ($gDW.m^{-2}$)).

		Mondego		Tagus	
		Gala	**Jusante**	**Pancas**	**Corroios**
Leaves	**Jun**	1132 ± 519	243 ± 47	146 ± 9	112 ± 2
	Aug	539 ± 173	365 ± 62	248 ± 37	137 ± 13
	Oct	683 ± 174	376 ± 97	248 ± 36	103 ± 2
	Dec	270 ± 32	158 ± 41	185 ± 25	105 ± 4
	Feb	642 ± 194	139 ± 31	84 ± 7	109 ± 6
	Apr	553 ± 102	276 ± 65	94 ± 9	76 ± 4
Stems	**Jun**	2064 ± 1372	1084 ± 130	118 ± 9	112 ± 4
	Aug	2329 ± 893	1201 ± 506	237 ± 21	152 ± 15
	Oct	3527 ± 430	1939 ± 527	238 ± 17	93 ± 1
	Dec	2131 ± 501	1401 ± 276	163 ± 8	107 ± 6
	Feb	2958 ± 938	924 ± 181	83 ± 10	110 ± 16
	Apr	1556 ± 453	957 ± 58	96 ± 9	117 ± 11
Belowground	**Jun**	1404 ± 591	3250 ± 633	826 ± 79	7189 ± 124
	Aug	1332 ± 642	3596 ± 1077	1173 ± 184	4671 ± 77
	Oct	1320 ± 172	3608 ± 723	1194 ± 225	3699 ± 283
	Dec	850 ± 217	2871 ± 581	804 ± 115	4506 ± 273
	Feb	1745 ± 219	2800 ± 891	527 ± 24	4851 ± 150
	Apr	966 ± 271	2737 ± 425	670 ± 54	5452 ± 167

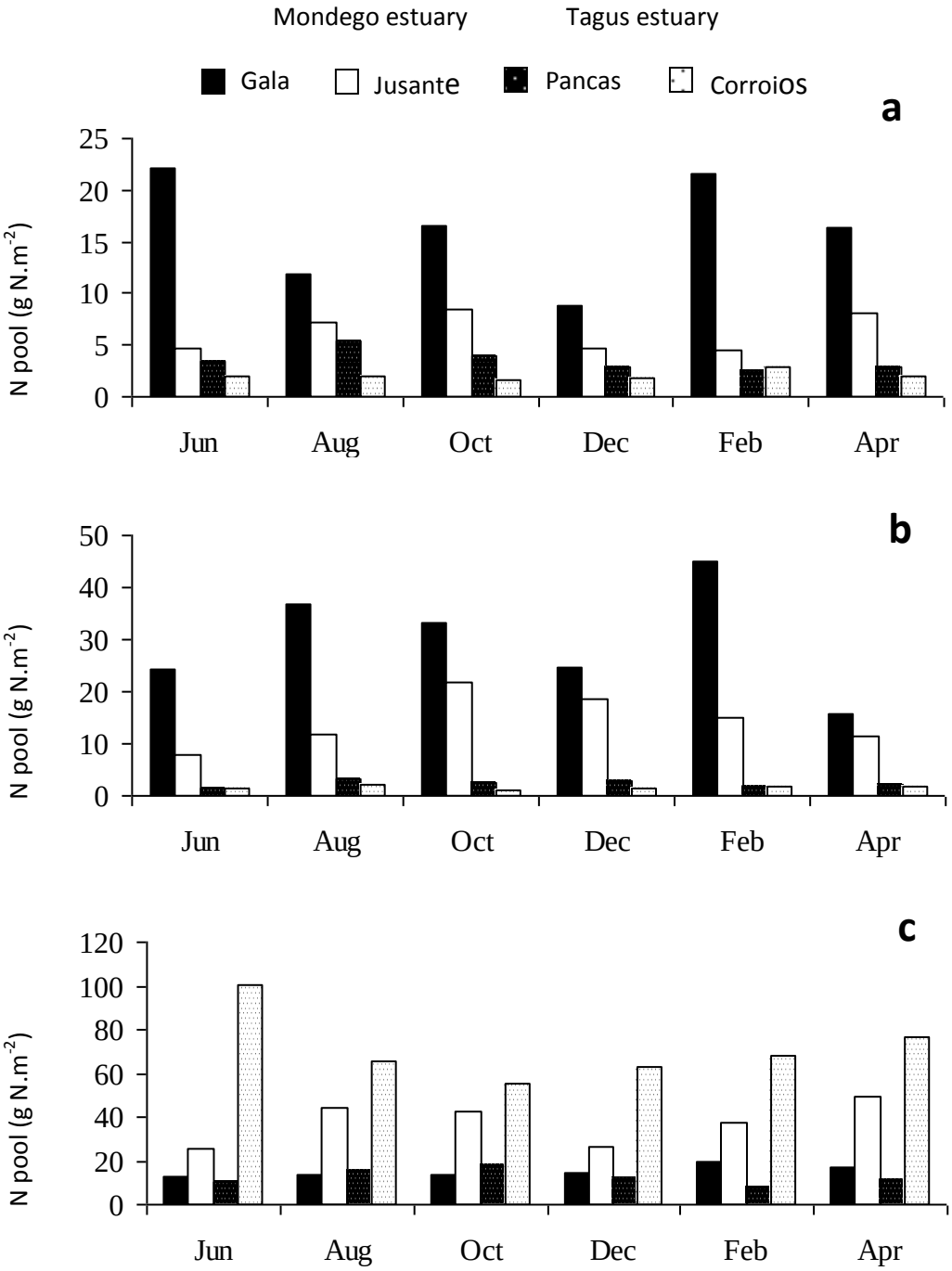


Figure 3. Nitrogen pool ($gN.m^{-2}$) (Biomass x N concentration) of *Spartina maritima* leaves (a), stems (b) and belowground biomass (c) in the salt marshes of the Mondego and Tagus estuaries for a one-year period.

Biomass and nitrogen productions

Gala and Corroios presented the highest total (above plus belowground) biomass production (3728 and 3610 gDW.m^{-2}.yr^{-1}) of *Spartina,* followed by Jusante (2059 gDW.m^{-2}.yr^{-1}) (Figure 4a). The lowest *Spartina* biomass production occurred at Pancas (986 gDW.m^{-2}.yr^{-1}). The relative proportion of aboveground/belowground biomass production varied largely when considering all the studied salt marshes. While at Gala, the majority (76%) of *S. maritima* biomass derived from aboveground part (leaves and stems), the opposite situation was registered at Corroios, wherein only 3% of the total biomass derived from leaves and stems of *S. maritima* (Figure 4a). Jusante presented a 1:1.5 and Pancas a 1:2 above:below biomass production ratio. In general, in the Mondego salt marshes, *S. maritima* was major represented by aboveground biomass, while in Tagus it was the belowground biomass that largely contributed to the total biomass production (Figure 4a). Gala, Jusante and Pancas presented a relatively similar belowground biomass production of *Spartina*, varying the aboveground biomass production. On the contrary, Corroios presented an extremely higher belowground biomass production, comparing with the aboveground production (Figure 4a).

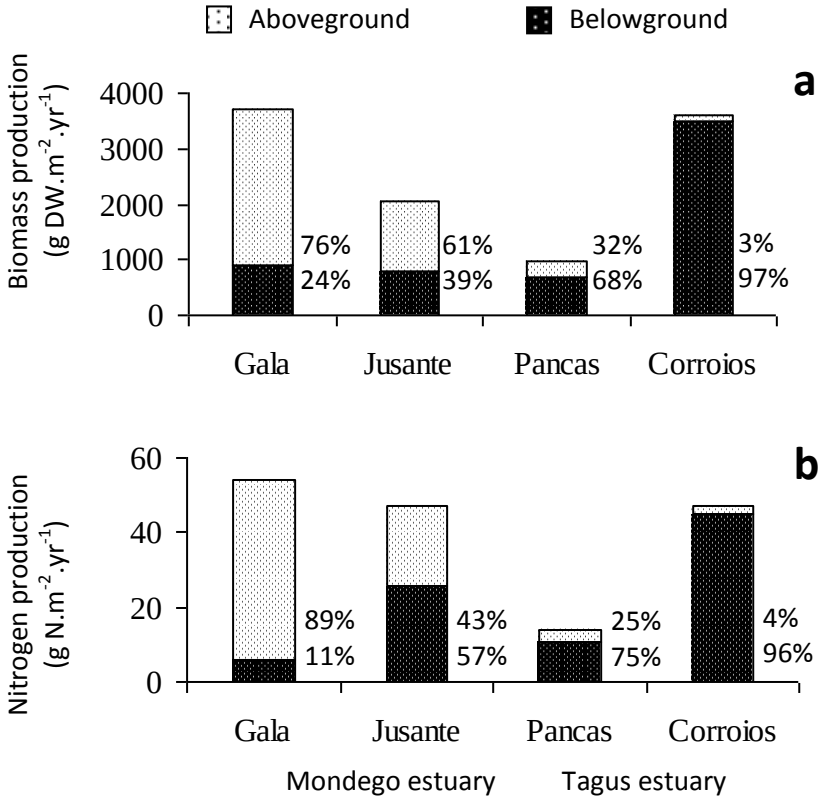


Figure 4 - Biomass and nitrogen production for aboveground and belowground parts of *Spartina maritima* at the salt marshes of the Mondego and Tagus estuaries.

Considering N production, *Spartina* from Jusante, Pancas and Corroios presented higher belowground production when compared to aboveground production (Figure 4b). On the other hand, at Gala 11% of *Spartina* N production is from belowground and 89% from aboveground material. It can clearly be observed that there is a tendency from Mondego to Tagus, more specifically from Gala, Jusante to Pancas and Corroios, to improve the belowground nitrogen production of *S. maritima*, rather than from aboveground vegetation.

According to the highest total biomass production of *Spartina* at Gala and Corroios, these sites presented the highest nitrogen pools in the plant (54.2 and 46.9 gN. $m^{-2}.yr^{-1}$) (Figure 4b). Pancas presented both the lowest biomass and nitrogen production (14.1 gN. $m^{-2}.yr^{-1}$) and Jusante showed an intermediate total biomass production, but an equal nitrogen production to Corroios (46.9 gN. $m^{-2}.yr^{-1}$). Thus, the same amount of nitrogen produces lower *Spartina* biomass at Jusante, when compared to Corroios, demonstrating lower N use efficiency.

At Gala, the turnover rate for above and belowground biomass is similar (Figure 5). Considering aboveground biomass Pancas presented the highest turnover and Corroios the lowest one (0.66 and 0.33 yr^{-1}, respectively) (Figure 5). Turnover rate for belowground biomass was similar between Gala, Pancas and Corroios (about 0.50 yr^{-1}) and lower at Jusante (0.22 yr^{-1}) (Figures 5 and 6). Turnover rate for nitrogen was lower at Gala in the belowground material and at Corroios in the aboveground part of *Spartina.* As a whole, Pancas presented the highest turnover rates and Corroios the lowest ones (Figure 5), meaning that younger salt marshes presented higher turnover rates.

Detritus production: biomass and nitrogen pool

Only 3.5 to 5.3% of the *Spartina* biomass production was retained as detritus, and all the rest (94.8 to 96.5%) did not rest there, being exported from the salt marsh (Figure 5). The Mondego salt marshes presented low biomass retention as detritus, with slightly higher values of detritus retention occurring in the Tagus salt marshes. In view of aboveground *Spartina* nitrogen production, the highest detritus nitrogen pool retained in the estuary occurred in the Tagus salt marshes (4.2 and 4.1% at Pancas and Corroios, respectively). At Gala and Jusante, only 1.9 and 0.8%, respectively, of the total nitrogen aboveground production stays in the salt marsh as detritus; all the rest is exported from the salt marsh to adjacent areas (Figure 5). Although Tagus retains slightly more nitrogen as detritus than Mondego, this is a slight difference in the nitrogen pool, with all salt marsh systems exporting an extremely large amount of annually produced nitrogen by *S. maritima*.

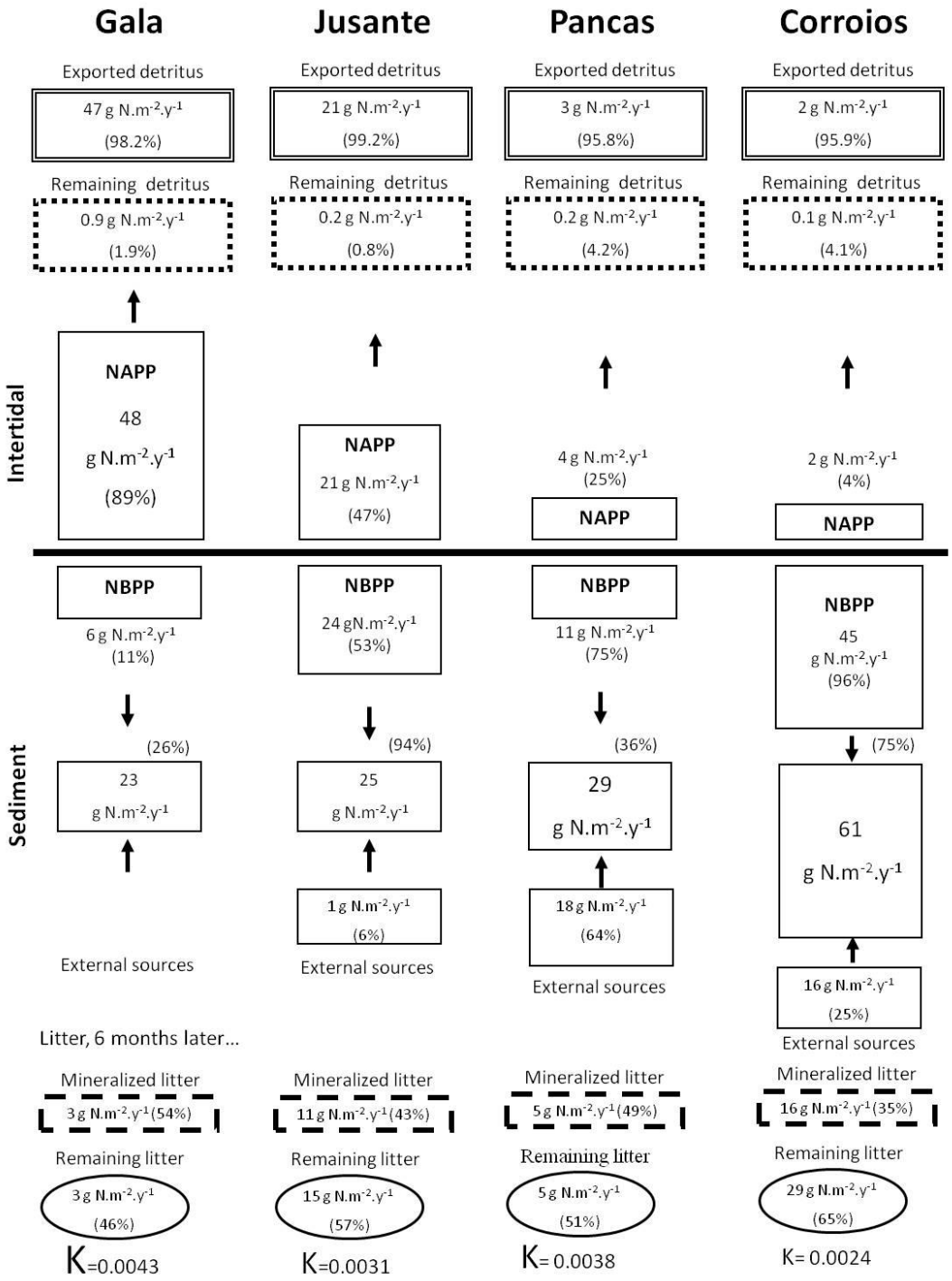


Figure 5 - *Spartina maritima* biomass and N productions, detritus moved by tides, decomposition of belowground material after certain period and nitrogen retention in the sediment of the salt marshes of the Mondego estuary.

Decay rate – Litterbag field experiment

S. maritima decomposition rate (velocity at which belowground material decomposes) varied among salt marshes (Table 3).

Table 3 – Decomposition rates (k) for *Spartina maritima* belowground biomass at Mondego and Tagus salt marshes during a 180 days period, according to the equation $X_t=X_0*e^{-kt}$. X_t is remaining dry weight in the litterbags (%) (see Figure 6), X_0 is initial dry weight and t is time in days.

Mondego				**Tagus**			
Gala		**Jusante**		**Pancas**		**Corroios**	
t (d)	**k**	**t (d)**	**k**	**t (d)**	**k**	**t (d)**	**k**
22	0.0087	22	0.0123	31	0.0179	31	0.0076
43	0.0087	43	0.0059	59	0.0045	59	0.0032
71	0.0081	71	0.0053	87	0.0068	87	0.0027
99	0.0061	99	0.0049	118	0.0038	118	0.0024
134	0.0036	134	0.0038	150	0.0038	150	0.0017
183	0.0043	183	0.0031	180	0.0038	180	0.0024

Gala and Pancas presented the highest decay rate after 6 months for *Spartina* belowground biomass (k=0.0043 and 0.0038, respectively), presenting about 50% of total biomass decomposed (Figure 6). Corroios presented the lowest decay rate (k=0.0024) at this time, with 65% of biomass still to decompose. Thus, at Corroios the belowground biomass decomposition is a slower process. Jusante presented 57% of the initial biomass after 6 months decomposing and a k=0.0031. Considering the previous results and extrapolating them to the belowground N production at all salt marshes, it can be observed that, for example after 6 months, Gala and Pancas constitute the sites with more N mineralized (54 and 49%, respectively), and Corroios, the salt marsh wherein still remains in form of litter 65% of the belowground N production (Figure 5).

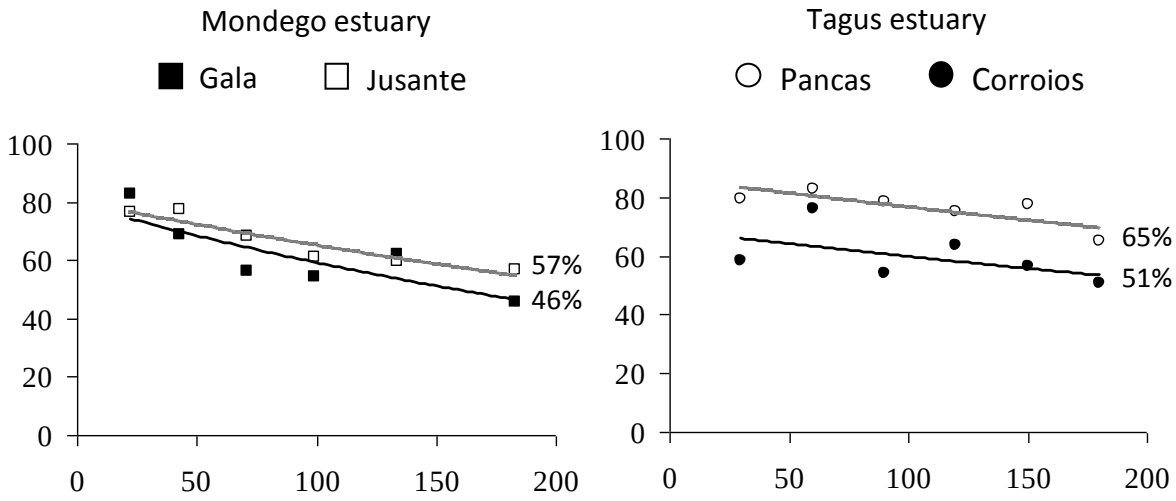


Figure 6. Remaining belowground material of *Spartina maritima* (dry weight - % (Xt)) over 180 days of decomposition in the litter bags.

Nitrogen retention in the salt marsh sediments

Nitrogen yearly retained in the sediments was calculated for all salt marshes (Figure 5). It was quantified considering both the sedimentation rate and the N content in the upper 5 cm of *Spartina* rooted sediment (Table 4). Mondego salt marshes present similar values of N annually stored in the sediment (3.24 and 3.63 mg N.g DW, respectively), and Pancas (Tagus) present slightly lower values (2.93 mg N.g DW). The nitrogen retained in the sediments of Corroios salt marsh is 2 to 3 fold higher (6.13 mg N.g DW), when compared to the other salt marshes (Table 4).

Table 4 – N annually retained in the salt marsh sediments of the Mondego and Tagus salt marshes, concerning sedimentation rate and N content in the upper 5 cm of *Spartina* rooted sediment.

	Salt marsh			
	Gala	**Jusante**	**Pancas**	**Corroios**
Sedimentation rate ($cm.yr^{-1}$)	0.7 [(a)]	0.7 [(a)]	1.0 [(b)]	1.0 [(b)]
Sediment N (mgN.gDW) (average±SD; N=12)	3.24±0.74	3.63±1.02	2.93±0.23	6.13±0.25
N retained ($gN.m^{-2}.yr^{-1}$)	22.7	25.4	29.3	61.3

(a) Extrapolated from Castro (2005).

(b) in: Caçador et al. (2007).

Spartina belowground production and also external sources are the contributors to the nitrogen stored in the salt marsh sediments. This N with an outside origin can be quantified subtracting the N produced by *Spartina* from the N annually retained in the sediment. Thus, at Jusante the greater percentage (94%) of N content in the sediment comes from the *Spartina* belowground system (Figure 5). Gala and Pancas presented 26 and 36% of total nitrogen retained in the sediment resultant from *Spartina* production (Figure 5). On the contrary, 75% of the N in Corroios rooted sediments results from the *Spartina* belowground production and only 25% of the N comes from external sources.

Discussion

Mondego salt marshes presented higher aboveground biomass of *S. maritima* when compared to belowground biomass, whereas Tagus salt marshes, mainly Corroios, presented higher belowground biomass production when compared to aboveground biomass. This high belowground/aboveground biomass ratio has already been described as a result of stressing environmental conditions for *Phragmites australis* in the Po Delta, Italy (Scarton et al., 1998 (1999)); and also for the seagrass *Zostera capensis* at Mozambique (De Boer, 2000). It is already known that environmental stress leads plants to attempt adaptation by investing in the belowground biomass (Groenendijk and Vink-Lieavaart, 1987) and that these plants under unfavourable soil conditions need a greater root surface (see Caçador et al., 1999). Thus, the increasing of the below: aboveground ratio from Gala and Jusante to Pancas and Corroios, may indicate an increase in physiological stress of *Spartina* on this way. Several factors, namely soil salinity, tidal inundation, nutrient availability, sediment type and drainage, may affect primary production of salt marsh plants (Ibañez et al., 1999, 2000). Thus, physical and chemical characteristics of the rooted sediments are important in determining plants productivity. Several authors have pointed out salinity as a determinant factor in the salt marsh plants productivity, with lower salinity values favouring the marsh plants productivity (Ibañez et al., 1999, 2000; Curcó et al., 2002; see Edwards and Mills, 2005).

Considering the mentioned above, the high sediment salinities and the fact that Corroios is an older salt marsh may explain the productivity of *S. maritima* at this site. Moreover, Corroios is the most stressing site of this study, concerning pollution as well as urban and industrial discharges from the nearest and densely populated city (Lisbon). Thus, *S. maritima* seems to reduce the aboveground production due to these stressing conditions and strongly invest on the belowground material. Edwards and Mills (2005) showed that the belowground

production of *Spartina alteniflora* increased with the increasing age of the created salt marshes in Louisiana (USA), accompanied by a tendency to decrease the aboveground biomass production. Accordingly, Pancas is a younger salt marsh which may explain the higher percentage of aboveground biomass than Corroios, and consequently the higher belowground production at Corroios. Gala and Jusante are younger salt marshes, concerning its characteristics of much open water and relatively simple tidal outlets to the sea, comparing with Corroios which presents channels relatively more complex (Valiela et al., 2000). In young salt marshes, concerning physical and chemical characteristics, the competition for nutrients is reduced/low, which in turn reduces the amount of belowground material vital for the plant (Caçador et al., 1999), explaining the higher aboveground biomass production in the youngest salt marshes.

It can clearly be observed that there is a tendency from Mondego to Tagus, more specifically from Gala, Jusante to Pancas and Corroios, to improve the belowground nitrogen production of *S. maritima*, rather than from aboveground vegetation (percentage), as well as occurred with biomass production, which may once more be explained by the increase in stressing environmental conditions present at each salt marsh.

Spartina detritus that remains near the plant represent a very small percentage of the aboveground productivity of this marsh plant (3.5 to 5.3%). The other part of detritus produced by the plant (94.8 to 96.5%) is exported to other areas of the salt marsh, to the estuary or even to the coastal waters. According to some authors, mature salt marshes (such as Corroios) export more material than the youngest ones, since as they mature and fulfil with sediments and plants, increase the exportation (Valiela et al., 2000). Although Corroios is the salt marsh that exports lower percentage of *Spartina* aboveground biomass production (94.8%), it can be observed that the difference in detritus exportation is very slight between marshes, which may allow us to conclude that there are no differences among the studied (young and mature) salt marshes.

Considering the total aboveground production, the export of N by detritus transport is probably significant for the N balance of the salt marsh. Thus, the mineralization of *Spartina* derived organic matter, and consequently the N cycling, almost entirely takes place outside the salt marsh system, namely in the estuary or in the coastal areas, which is the opposite that has been described for other salt marshes (Hemminga et al., 1996; Bouchard and Lefeuvre, 2000). Nevertheless, the high detritus exportation is not surprising in the Mondego and Tagus salt marshes since the tidal inundation occurs twice a day, contributing to the wash out of detritus.

Decomposition rate of *S. maritima* varied among salt marshes, with Gala and Pancas presenting the highest decay rate for the belowground biomass after 6 months of

decomposition, and Corroios presenting the slower decay rate. At Gala and Pancas about half of the belowground biomass is mineralized within 6 months, remaining the other 50% in the sediment as litter. At Corroios after the same 6 months, 65% of the biomass still remains in the sediment. This slower decomposition process in Corroios indicates a tendency for higher accumulation of *Spartina* derived organic matter in the sediments of this salt marsh, thus a higher N retention in these sediments. Villar et al. (2001) also reported the net accumulation of macrophyte detritus due to high macrophyte production and low decomposition rates. Differences in decomposition rate have frequently been explained by differences in the chemical composition of the litter (N, P and C content) and tidal action along the elevation gradient of the salt marsh (see De Boer, 2000; see Bouchard and Lefeuvre, 2000; see Villar et al., 2001). The chemical composition of the litter is not considered in this work but may be an important aspect to consider in future works. Considering the fact that Corroios is an older salt marsh, it may be hypothesized that this salt marsh may be in a higher elevation than the others. Thus, the tidal flushing may be lower, which may reduce the decomposition kinetics at this site, since this aspect seems important in maintaining litter moisture (see Bouchard and Lefeuvre, 2000) and consequently in accelerating decomposition. According to some authors (see De Boer, 2000) the higher organic matter content in the sediment, and the higher clay content (which increases the water retention capacity) favours decomposition. However, at Corroios there is high organic matter content (27%) and high clay content but the decomposition rate was slow. One possible explanation can be the variation of influence of these parameters along the year.

Nitrogen stored in the studied salt marsh sediments results from *Spartina* belowground production and also from external sources (namely sedimentation processes). From the total N quantified, for instance, in the Pancas salt marsh sediment, a very small amount (0.3%) corresponds to inorganic N (ammonium, nitrate and nitrite) and more than 99% of the total N corresponds to organic N (Cartaxana and Lloyd,1999). At Gala and Pancas the N in the sediment comes mainly from external sources, whereas at Jusante and Corroios the main percentage of N retained in the sediment comes from the belowground production of *S. maritima*. This makes sense since these latter salt marshes present lower decomposition rates and higher belowground N production, contributing to this higher N retention/accumulation in the sediment. According to some authors (Rozema et al., 2000), the increased N content in salt marsh soil with increasing age of the salt marsh indicates that the salt marsh acts as a sink for N, which can be stated to Corroios.

Conclusions

Overall, this study reveals some differences among all the studied salt marshes, with Corroios presenting the most far-away characteristics from the other three sites. Gala and Pancas had the highest aboveground biomass production, whereas at Corroios there was a huge belowground biomass production. This may be due to the highest environmental stressing conditions occurring in Corroios. The amount of detritus exported to the adjacent areas is similar between young and mature/old salt marshes and represents a huge percentage of aboveground *Spartina* primary production. Thus, this type of transport is probably significant for the N balance of the salt marshes. Litter decay was slower at Corroios indicating a tendency for higher accumulation of organic matter and N in these sediments, which actually was observed. Younger salt marshes had higher turnover rates of *S. maritima*. Lastly, high N content in *S. maritima* sediments of Corroios may allow to conclude this older salt marsh presents higher capacity to sequestrate N than the younger studied salt marshes.

To sum up, *S. maritima* salt marshes may trap N, removing its excess, and therefore help reducing eutrophication in the estuarine ecosystem. Nevertheless, this ability to remove N varies among salt marshes and estuaries, depending on its biotic and abiotic characteristics, namely considering young and mature/old salt marshes.

References

Bouchard, V., Lefeuvre, J.C., 2000. Primary production and macro-detritus dynamics in a European salt marsh: carbon and nitrogen budgets. Aquatic Botany 67, 23-42.

Caçador, I., Costa, A.L., Vale, C., 2004. Carbon storage in Tagus salt marsh sediments. Water, Air, and Soil Pollution , 701-714.

Caçador, I., Costa, A.L., Vale, C., 2007. Nitrogen Sequestration Capacity of Two Salt Marshes from the Tagus estuary. Hydrobiologia 587, 137-145.

Caçador, I., Mascarenhas, I., Mascarenhas, P., 1999. Biomass of Spartina maritima, halimione protulacoides and Arthrocnemum fruticosum in Tagus estuary salt marshes, in: Leith, H., Moschenko, M., Lohmann, M., Koyro, H-W., Hamby A. (Eds.), Halophyte uses in different climates I. Backhuys Publishers, Leiden, The Netherlands, pp. 33-41.

Cartaxana, P., Lloyd, D., 1999. N2, N2O and O2 Profiles in a Tagus estuary salt marsh. Estuarine, Coastal and Shelf Science 48, 751–756.

Castro, P.C.O., 1999. Senescência, decomposição e nutrição azotada em *Spartina maritima* no estuário do rio Mondego. Master Thesis, University of Coimbra, Portugal.

Castro, P.C.O., 2005. Assessing key-habitat loss due to eutrophication in the Mondego and Mira estuaries. PhD Thesis, University of Coimbra, Portugal.

Coelho, J.P., Flindt, M.R., Jensen, H.S., Lillebø, A.I., Pardal, M.A., 2004. Phosphorus speciation and availability in intertidal sediments of a temperate estuary: relation to eutrophication and annual P-fluxes. Estuarine, Coastal and Shelf Science 61, 583-590.

Constanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg K., Naeem, S., O' Neill, R.V., Paruelo, J., Raskin, R.G., Sutton P., van den Belt, M.,1997. The value of the world's ecosystem services and natural capital. Nature 387, 353-360.

Curcó, A., Ibañez, C., Day, J.W., Prat, N., 2002. Net primary production and decomposition of salt marshes of the Ebre Delta (Catalonia, Spain). Estuaries 25, 309-324.

De Boer, W. F., 2000. Biomass dynamics of seagrasses and the role of mangrove and seagrass vegetation as different nutrient sources for an intertidal ecosystem. Aquatic Botany 66, 225-239.

De la Cruz, A.A., Hackney, C.T., 1977. Energy value, elemental composition, and productivity of belowground biomasa of a *Juncus* tidal marsh. Ecology 58, 1165-1170.

Edwards, K.R., Mills, K.P., 2005. Aboveground and belowground productivity of *Spartina arteniflora* (smooth cordgrass) in natural and created Louisiana salt marshes. Estuaries 28, 252-265.

Eyre, B.D., Ferguson, A.J.P., 2002. Comparison of carbon production and decomposition, benthic nutrient fluxes and denitrification in seagrass, phytoplankton, benthic microalgae- and macroalgae-dominated warm-temperate Australian lagoons. Marine Ecology Progress Series 229, 43-59.

Foote, A.L., Reynolds, K.A., 1997. Decomposition of saltmeadow cordgrass (*Spartina patens*) in Luoisiana coastal marshes. Estuaries 20, 579-588.

Gameiro, C., Cartaxana, P., Cabrita T., Brotas, V., 2004. Spatial and Temporal Variability in the Phytoplankton Composition of an Estuarine System. Hydrobiologia 525, 113-124.

Groenendijk, A.M., Vink-Lieavaart, M.A., 1987. Primary production and biomass on a Dutch salt marsh: emphasis on the belowground component. Vegetatio 70, 21-27.

Hemminga, M.A., Cattrijsse, A., Wielemaker, A., 1996. Bedload and nearbed detritus transport in a tidal saltmarsh creek. Estuarine, Coastal and Shelf Science 42, 55-62.

Herbert, R.A., 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews 23, 563-590.

Ibañez, C., Curcó, A., Day Jr, J.W., Prat, N. 2000. Structure and productivity of microtidal Mediterranean coastal marshes, in: Weinstein M.P., Kreeger, D.A., (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, pp. 107-136.

Ibañez, C., Day Jr., J.W, Pont, D., 1999. Primary production and decomposition of wetlands of the Rhône Delta, France: Interactive impacts of human modifications and relative sea level rise. Journal of Coastal Research 15, 717-731.

Jickells, T., 2005. External inputs as a contributor to eutrophication problems. Journal of Sea Research 54, 58-69.

Lillebø, A.I., Neto, J.M., Martins, I., Verdelhos, T., Leston, S., Cardoso, P.G., Ferreira, S.M., Marques, J.C., Pardal, M.A., 2005. Management of a shallow temperate estuary to control eutrophication: the effect of hydrodynamics on the system nutrient loading. Estuarine, Coastal and Shelf Science 65, 697-707.

Limnologisk Metodik. 1992. Ferskvandsbiologisk Laboratorium. Københavns Universitet (Ed.). Akademisk Forlag. København. 172 pp.

Maricle, B.R., Lee, R.W., 2002. Aerenchyma development and oxygen transport in the estuarine cordgrasses *Spartina arteniflora* and *S. anglica*. Aquatic Botany 74, 109-120.

McGlathery, KJ., Sundbäck, K., Anderson, I.C., 2004. The importance of primary producers for benthic nitrogen and phosphorus cycling, in: Nielsen, S., Banta, G., Pedersen, M., (Eds.), Estuarine nutrient cycling: The influence of primary producers. Kluwer Academic Publishers, The Netherlands, pp. 231-261.

McLusky, D.S., Elliot, M., 2004. The Estuarine Ecosystem - Ecology, Threats, and Management, 3rd ed. Oxford University Press.

National Research Council (NRC), 2000. Clean Coastal Waters: Understanding and reducing the effects of Nutrient Pollution, Washington, DC, 165-176.

Nedwell, D.B., Jickells, T.D., Timmer, M., Sanders, R., 1999. Nutrients in estuaries, in: Nedwell, D.B., Raffaelli, D.G., (Eds), Estuaries. Advances in Ecological Research 29, 43-92.

Nixon, S.W., 1980. Between coastal marshes and coastal waters-a review of twenty years of speculation and research on the role of salt marshes in estuarine productivity and water chemistry, in: Hamilton, P. Macdonald, K.B., (Eds.), Estuarine and Wetland Processes with Emphasis on Modelling. Plenum Press, New York, pp. 437-525.

Odum, W.E., Odum, E.P., Odum, H.T., 1995. Nature's pulsing paradigm. Estuaries 18, 547-555.

Pedersen, M.F., Nielsen, S.L., Banta, G.T., 2004. Interactions between vegetation and nutrient dynamics in coastal marine ecosystems: an introduction, in: Nielsen, S., Banta, G., Pedersen, M., (Eds.), Estuarine nutrient cycling: The influence of primary producers. Kluwer Academic Publishers, The Netherlands, pp. 1-15.

Scarton, F., Day, J.W., Rismondo, A., 1998(1999). Above and belowground production of *Phragmites australis* in the Po Delta, Italy. Boll. Musc. civ. St. nat. Venezia 49, 213-222.

Valiela, I., Bowen, J.L., 2002. Nitrogen sources to watersheds and estuaries: role of land cover mosaics and losses within watersheds. Environmental Pollution 118, 239-48.

Valiela, I., Cole, M.L., Mcclelland, J., Hauxwell, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes, in: Weinstein, M.P., Kreeger, D.A., (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, 23-38.

Villar, C.A., de Cabo, L., Vaithiyanathan, P., Bonetto, C., 2001. Litter decomposition of emergent macrophytes in a floodplain marsh of the Lower Paraná River. Aquatic Botany 70, 105-116.

Vitousek, P.M., Aber, J., Bayley, S.E, Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, G.D., 1997. Human alteration of the global nitrogen cycle: Causes and consequences. Ecological Applications 7, 737-750.

Widdows, J., Brinsley, M., 2002. Impact of biotic and abiotic processes on sediment dynamics and the consequences to the structure and functioning of the intertidal zone. Journal of Sea Research 48, 143-156.

3. Denitrification in *S. maritima* salt marshes: a contribution to reduce eutrophication as a service provided in salt marshes

Abstract

In the present study we hypothesize that comparatively to sediments without vegetation salt marshes rhizosediment enhance denitrification rates, i.e. N_2 removal. Thus, this study was done seasonally in a *Spartina maritima* salt marsh and in adjacent mudflats without vegetation, in order to estimate denitrification rates (^{15}N-isotope pairing technique), potential nitrification and nutrient fluxes. *Spartina maritima* controlled the efflux of ammonium (NH_4-N) during spring/summer, especially during the day and there was always a net uptake of NO_x-N. Denitrification rates and nutrient flux measurements in this study were highly variable, as has been documented in other works. Potential nitrification rates were significantly higher in autumn and winter and there were no statistically significant differences between bare bottom and *Spartina maritima* vegetated sediment. Results show that denitrification rates in sediment without vegetation (maximum rate 151 ± 24 μmol $N_2m^{-2}h^{-1}$ (avrg ± SD) (summer, dark) were within the range of other comparable systems. In *Spartina maritima* vegetated sediment denitrification rates were generally higher than values recorded in Venice lagoon. Seasonally, denitrification rates were significantly higher in winter under dark conditions (676 ± 497 μmol $N_2m^{-2}h^{-1}$) (avrg ± SD). When compared to sediments without vegetation, denitrification rates in *Spartina maritima* salt marshes, was significantly higher in winter. This can potentially contribute to a higher reduction of the land driven nitrogen inputs to the Tagus estuary at this time of the year, contributing to the reduction of nitrate availability in the following spring.

Keywords: Denitrification, Salt marshes, Ecosystem services, Eutrophication, *Spartina maritima,* ^{15}N-isotope pairing technique

Introduction

Salt marshes are greatly important as N (nitrogen) sinks through plants' biomass production (i.e. the N incorporation in standing biomass, detritus, litter and sediments) (Bouchard and Lefeuvre, 2000; Edwards and Mills, 2005; Caçador et al., 2007; Sousa et al., 2008) and through denitrification (e.g. Teal and Howes 2000; Valiela and Cole, 2002), which counteracts eutrophication in coastal areas (Seitzinger, 1988). In fact, according to Galloway (1998) review, and considering non-human-impacted environments, most of the land-derived nitrogen loads to coastal environments could be denitrified in estuarine and shelf regions. Nevertheless, anammox (anaerobic ammonium oxidation) also contributes to N removal in aquatic ecosystems (Trimmer et al., 2003), although in estuaries denitrification seems to be the most significant process that produces N_2 (Schlesinger, 1997; Jaffe, 2000). The role of anammox to N removal depends greatly on water depth and mineralization rates, being much lower in estuaries than in marine wasters (Dalsgaard et al., 2005). In estuarine shallow waters the organic enrichment of sediments seems to increase denitrification in a greater extent than anammox (Dalsgaard et al., 2005). Namely in the Thames estuary anammox contributed with less that 10% for N_2 production (Trimmer et al., 2003), while Risgaard-Petersen et al. (2004) recorded that comparatively to marine deep sea sediments, where annamox represented 30 to 70% of N_2 production, in estuaries it represented 5 to 24 %. Some of the physicochemical factors influencing denitrification rates in aquatic ecosystems are nitrate concentration, easily degradable organic carbon, oxygen availability, temperature, light, sulfide concentration, and water retention time (Seitzinger, 1988; Cornwell et al., 1999; Piña-Ochoa and Álvares-Cobelas 2006; Silvennoinen et al., 2008). In addition there are also biological factors that may affect denitrification rates, namely plant roots, fauna through bioturbation and bio-irrigation and the microbiological abundance and activity. Plants can influence denitrification rates (Reddy et al., 1989; Howarth et al., 1996; Cornwell et al., 1999; Erickson et al., 2003; Piña-Ochoa and Álvares-Cobelas, 2006; Koop-Jacobsen and Giblin, 2009) due to O_2 diffusion through the aerenchyma (Maricle and Lee, 2002) and creation of oxic micro-zones surrounding the roots and rhizomes (the rhizosphere) at a certain depth in the sediment, which enhances coupled nitrification-denitrification. *Spartina maritima* also create a more oxidized rhizosphere, which enhances sulfide oxidation and contributes to sulfide detoxification (Madureira et al., 1997). It has been shown that bioturbation by benthic macrofauna significantly stimulates *in situ* sediment denitrification, i.e., the stimulation of denitrification, which is associated with the sediment layer where the infauna is most active, can occur at different depths in the sediment

(Gilbert et al., 1998). Lastly, plants and microphytobenthos (MPB) compete with denitrifier microbial community for substrate (nitrate), which can influence as well denitrification rates.

Nitrification (the microbial aerobic oxidation of NH_4^+ and NO_2^- to NO_3^-) is an important step of the nitrogen cycle that occurs in the oxic surface sediment. The product of this process (NO_3^-) is later on denitrified (coupled nitrification-denitrification (D_n)) but can also diffuse from the sediment to the water column. Since many biotic and abiotic factors may influence potential nitrification (e.g. plant roots, fauna abundance and activity of nitrifying bacteria, and temperature, oxygen penetration, NH_4^+ concentration, (Henrikson et al., 1981)), it is important to quantify potential nitrification to estimate nitrifier microbial community and preview D_n.

Several works have been performed in order to quantify denitrification in different aquatic ecosystems, namely freshwater tidal marshes (Seitzinger, 1988; Cornwell et al., 1999), estuaries, rivers, lakes, coastal waters (Steingruber et al., 2001; Piña-Ochoa and Álvares-Cobelas, 2006), and wetlands (Merrill and Cornwell, 2000; Risgaard-Petersen, 2003; Trimmer et al., 2003; Sundbäck et al., 2006). However, regarding denitrification in estuaries, there are some works concerning mud flats (e.g. Cabrita and Brotas, 2000; Risgaard-Petersen, 2003; Sundbäck et al., 2006) and much less concerning salt marshes (Valiela and Teal, 1979; Koch et al., 1992; White and Howes, 1994; Erickson et al., 2003; Poulin et al., 2007). Within these studies, different techniques have been applied, which may restrict the comparison between systems.

This work intended to evaluate the role of *S. maritima* salt marshes on denitrification, as a service provided by the ecosystem. To do so, we hypothesize that in salt marshes sediments the presence of plant detritus can enhance denitrification; as well, the rhizosphere environment may enhance nitrification and denitrification at depths were roots are more active; and that in eutrophic systems, were nutrients are not limiting, marsh plants and bacteria do not compete for resources. Thus, we hypothesize that comparatively to sediments without vegetation salt marshes rhizosediment enhance denitrification rates, i.e. N_2 removal.

Methods

Sampling site and procedure

Sampling took place in the Tagus estuary (Figure 1, case study 2, chapter 1, page 56), located in the southern European Atlantic margin (Portugal). Tagus estuary is one of the biggest European estuaries (320 km^2), classified by the Convention of Wetlands as a Ramsar site. It is characterized by water temperature ranging between 20-26 °C in the summer and 8-

18 °C in the winter (Gameiro et al., 2007). Water column DIN (dissolved inorganic nitrogen) concentrations, for the period 1999 and 2005, varied seasonally between 27 ± 19 $\mu mol.l^{-1}$ (avrg ± SD) in summer to 84 ± 33 $\mu mol.l^{-1}$ (avrg ± SD) in winter; PO_4-P concentrations vary between 3.4 ± 1.1 $\mu mol.l^{-1}$ (avrg ± SD) during winter and 4.5 ± 3.0 $\mu mol.l^{-1}$ (avrg ± SD) in autumn (Gameiro et al., 2007).

S. maritima is an herbaceous perennial plant that colonizes estuarine intertidal mudflats and is distributed in the coasts of western, southern and southeastern Europe, and also in western Africa. It is one of the most common halophytes colonizing salt marshes in the Tagus estuary, which has 20 km^2 of salt marsh vegetation (Simas et al., 2001). *S. maritima* is the dominant species in the lower marsh, with a coverage area of 675 ha, which represent 1/3 of the total marsh area (Simas et al., 2001; Reboreda and Caçador, 2007). It is described as a pioneer species, tolerating high salinity and "long" flooding conditions common in low marshes. At this system, *S. maritima* aboveground biomass is 0.60 ± 0.02 $kgDW.m^{-2}$ while the belowground biomass is 3.60 ± 0.15 $kgDW.m^{-2}$ (Reboreda and Caçador, 2007).

A seasonal study was performed from autumn 2007 to summer 2008. Sampling was performed in spring tides during low tide. Ten sediment cores were collected in the *S. maritima* salt marsh (each core containing one or two shoots of *S. maritima* – inter-core plant biomass was as similar as possible) using a Plexiglass core (∅ 8 cm; 30 cm height). Each sediment core was 15 cm depth. Additional five sediment cores (5 cm depth) were collected in order to characterize the vegetated sediment. The same number and type of sediment cores were collected in the adjacent sediment without vegetation. Estuarine water was collected in containers and taken to the laboratory to be used in the incubation procedure. Water and sediment *in situ* temperature were recorded and all the samples were immersed in estuarine water, and taken to the laboratory (within 1 h). The *in situ* temperature conditions were maintained using coolers.

Sediment characterization

Sediment was characterized for microphytobenthos Chlorophyll (Chl) *a* and sediment particle size. For Chl *a* determination, the top 5 mm of the five sediment cores (5 cm depth) was removed, weighed and stored at -80 °C. Later on, the sediment was freeze-dried and a 0.3 g aliquot was immersed in 5 ml of 90% acetone and stored at -20 °C for 24 h. Afterwards, samples were stirred in the vortex, centrifuged for 10 min at 4000 rpm and supernatant was analyzed in a UV-1603 spectrophotometer. Calculations were done according to Lorenzen

(1967). Chl *a* was also estimated from the trichromatic equations of Jeffrey and Humphrey (1975) which does not include the acidification step.

Sediment particle size was determined by sequential sieving of the top 5 cm sediment cores and classified according to Folk (1954). Organic matter was quantified as loss on ignition (% LOI) during 8 h at 500 °C.

Potential nitrification measurements

Potential nitrification was measured through a slurry incubation experiment, according to Hansen (1980, in Rysgaard et al., 1994) in *S. maritima* vegetated sediment (n = 5) and adjacent sediment without vegetation (n = 5). Homogenised surface sediment aliquots (0 – 1 cm depth; 2 ml) were incubated with 20 mM NH_4Cl and 4 mM KH_2PO_4 in 40 ml artificial seawater adjusted to *in situ* salinity (ASW), Incubations were done at ambient temperature, dark conditions, and continuous shaking. Samples for determination of nitrification rates were taken at timed intervals of 1 h, over 5 h. Samples were centrifuged (8 min at 3000 rpm) and supernatant water sample was filtered and frozen for later NO_x-N (NO_3-N +NO_2-N) analysis. NO_x-N concentration were expected to increase over time (5 h of incubation) in a linear way, meaning that added NH_4-N is nitrified immediately after the beginning of the incubation. Potential nitrification was calculated from this increase of NO_x-N and according to Rysgaard et al. (1994).

Incubation procedure – Nutrient fluxes and O_2 consumption

Incubations were performed in a batch mode assay in a tank/incubator with ten cores each time (i.e. 5 cores with *S. maritima* and 5 cores of adjacent sediment without vegetation, meaning that light and dark incubations were always done in different cores). The cores were maintained aerated overnight (with an air pump and a magnetic stirrer rotating a magnet inside each core, as described at Cabrita and Brotas (2000) and Dalsgaard et al. (2000)) and under natural seasonal light-dark cycle. On the following day, each core was sealed with Plexiglass stoppers and incubations were done as described at Cabrita and Brotas (2000) and Dalsgaard et al. (2000). After measuring the nutrient fluxes cores stayed aerated overnight to re-establish the equilibrium between sediment and water column, and denitrification rates measured in the following day. Flux incubation time was calculated considering the reduction of O_2 concentration in the water column, which can't decrease more than 20% of the initial one. Nutrients (NH_4-N, NO_x-N,) and oxygen fluxes were calculated on a mass balance approach. Both, dark and light incubations for nutrient fluxes were done twice each season, using extra

cores, in order to increase the number of replicates, i.e. twice (5 light vegetated + 5 light without vegetation) and twice (5 dark vegetated + 5 dark without vegetation).

Nutrient and oxygen analyses

Dissolved oxygen was quantified by Winkler titration (Grasshoff et al., 1983). Inorganic nutrient concentrations were quantified in water samples previously filtered through GF/C Whatman filters and immediately frozen. Later on, colorimetric analyses in a Tecator FIAstar_5000 Analyser were performed. NO_3-N was quantified according to Grasshoff (1976), NO_2-N according to Bendschneider and Robison (1952). NH_4-N concentrations were determined using colorimetric methods in filtered samples according to Koroleff (1969/1970).

Denitrification rates' measurements

Denitrification rates were measured in the same cores as nutrient fluxes, according to the isotope pairing technique (Nielsen, 1992). Following the method, $^{15}NO_3$ (from a $Na^{15}NO_3$ stock solution, 99% Sigma Aldrich) was added to the estuarine water in the container with ten sediment cores each time (5 light vegetated + 5 light without vegetation and 5 dark vegetated + 5 dark without vegetation), to a final concentration of at least 20% of the O_2 concentration in the incubation water. Diffusion time for $^{15}NO_3$ was about 15 minutes and time was calculated according to Dalsgaard et al., (2000). The sediment cores were closed with PVC lids and incubation started. Incubation time was calculated according to the O_2 fluxes done the day before, so that the O_2 concentration never decreased 20% of the initial O_2. In the end of incubations, water samples were collected to exetainer vials (Exetainer, Labco, High Wycombe, UK) for N_2 analyses (200 μl of $ZnCl_2$ (50%, w/v) was added to stop bacterial/any biological activity). Water samples were filtered and stored for NO_3 analyses. Immediately after, each core was carefully mixed/slurried in order to homogenise the dissolved N_2 in the water column and in porewater and new samples for N_2 analyzes were collected. Thus, N_2 diffused to the water column during the incubation and N_2 still in porewater can be sampled and quantified. Denitrification rates were calculated according to Nielsen (1992).

^{15}N- IPT assumptions

The isotope pairing technique (IPT) has the following assumptions: 1) the added $^{15}NO_3$ does not affect the production of $^{14}N_2$; 2) the produced $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ is binomially distributed; and 3) $^{14}NO_3$ and $^{15}NO_3$ mixes homogenously in the nitrate reduction zone in the sediment. In order to test these assumptions, a $^{15}NO_3$ concentration series experiment was done following Nielsen (1992). Seven different $^{15}NO_3$ concentrations were tested (20 to 160

μM, in order to include a wide range of the water column NO_3 concentrations) and denitrification rates were quantified.

Plant biomass and fauna characterization

After all the incubations, *S. maritima* plants were carefully washed and rinsed with distilled water and then dried at 60 °C for dry weight (DW) quantification per sample. Sediment from each core, with and without vegetation, was sieved through a 500 μm mesh size net and macrofauna was collected, identified and species abundance calculated.

The biological factors (MPB), plant density and fauna abundance were considered because they can affect oxygen production and consumption and consequently other chemical processes such as mineralization, denitrification, and other nutrient fluxes (Rysgaard et al., 1995; Hulth et al., 2005; Sundbäck et al., 2006).

Statistical analysis

Linear correlation was performed (Pearson and Spearman rank correlations) to test for correlations between $^{15}NO_3$ concentration in the water column and D_w and D_n. Analyses were performed with SPSS 17.0 and with STATISTICA 9 software package. Two-way ANOVA was performed to test for differences in potential nitrification rates and in denitrification rates between dark/light conditions and *S. maritima* vegetated sediment/bare bottom. If needed, data were transformed to satisfy the ANOVA assumptions. Cochran's Q and Kolmogorov-Smirnov tests were used to analyse homogeneity of variances and normality of data, respectively. One-way ANOVA was performed to test for differences in D_t (total denitrification) between seasons.

Results

Sediment characterization, plant and macrofauna biomass

Temperature in water and in sediment showed a clear seasonal variation, with higher values in spring and summer and lower in autumn and winter. In both areas (with and without vegetation) the percentage of fine particles was higher in autumn/winter period with silt and clay ranging between 14% and 28% of the total. The percentage of organic matter (determined as percentage of loss on ignition, %LOI) was higher in vegetated sediment (12.9 to 17.5%) than in bare bottom (8.9 to 14.3%).

MPB abundance (estimated as concentration of Chl *a*) depended on the sediment type and season (there was interaction between both factors, Two-way ANOVA, $F = 4.55$, $p < 0.05$) and was comparatively higher in the *S. maritima* vegetated sediment. *S. maritima* dry weight per core ranged from 0.8 ± 0.2 to 2.2 ± 1.5 (g DW), with no clear seasonal variation (Table 1). In the cores from both sites, the more abundant infauna species were *Hydrobia ulvae, Scrobicularia plana, Hediste diversicolor* and *Abra tenuis,* and seasonally abundance was trendily higher in spring. In *S. maritima* vegetated sediment the mean macrofauna abundances were 1.1 to 2 times higher than in the sediment without vegetation.

Potential nitrification rates

Slurry incubations' salinity ranged between 28 and 30. Potential nitrification rates were significantly higher in winter and autumn when compared to spring and summer (Two-way ANOVA, $F = 11.99$, $p < 0.0001$) (Figure 1). There were no statistically significant differences between bare bottom and *S. maritima* vegetated sediment (Two-way ANOVA, $F = 0.49$, $p > 0.05$).

Table 1. Sediment characterization, *Spartina maritima* biomass and the macrofauna species abundance in each season. Fauna abundance means number of animals/individuals per m^{-2}.

			Autumn	Winter	Spring	Summer
Water temperature (^{0}C) (incubation)			18	18	23	26
Sediment	*In situ* temperature (^{0}C)	*Spartina*	14	16	24	24
		Bare bottom	15	17	23	26/28
	Granulometry	*Spartina*	Fine sand: 13%(63-125μm) 64%(>125 μm) Silt and clay: 14% (<63μm)	Fine sand: 10%(63-125μm) 62%(>125μm) Silt and clay: 28% (<63μm)	Fine sand: 18%(63-125μm) Silt and clay: 82% (<63μm)	Fine sand: 23%(63-125μm) 65%(>125μm) Silt and clay: 9% (<63μm)
		Bare bottom	Fine sand: 9%(63-125μm) 65%(>125μm) Silt and clay: 19% (<63μm)	Fine sand: 11%(63-125μm) 60%(>125μm) Silt and clay: 24% (<63μm)	Fine sand: 9%(63-125μm) 70%(>125μm) Silt and clay: 13% (<63μm)	Fine sand: 34%(63-125μm) 50%(>125μm) Silt and clay: 13% (<63μm)
	LOI (%)	*Spartina*	17.5	12.9	17.2	
		Bare bottom	10.8	11.8	14.3	8.9
	MPB Chlo a (avrg±SD, n=3 to n=5)	*Spartina*	71.9±16.2	46.4±20.5	60.2±15.8	41.9±7.3
		Bare bottom	10.6±2.6	7.9±1.6	21.5±1.4	12.7±1.6
Plant (g DW/core) (avrge±SD, n=15)		*Spartina maritima*	2.2±1.5	0.8±0.5	0.7±0.2	1.4±1.1

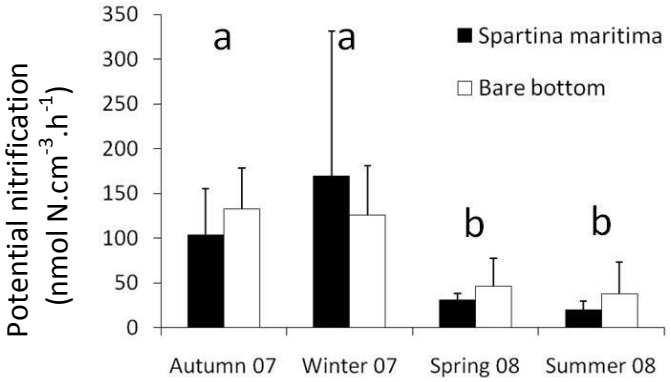


Figure 1. Potential nitrification in *S. maritima* vegetated sediment (black bars) and bare bottom (white bars), during autumn, winter, spring and summer (average ± SD, n = 5). Different lowercase letters (a, b) means statistical significant differences ($p < 0.05$).

O_2 and nutrient fluxes

Oxygen and nutrient concentrations at the beginning of the incubations varied seasonally (Table 2). In both areas (with and without vegetation) oxygen was usually net consumed under dark and light conditions (Figure 2). Nevertheless, under dark conditions, when respiration is not compensated by primary production, O_2 consumption was greater. Seasonally, variation is only observed in bare bottom, being the consumption higher in spring and summer together with higher MPB and fauna abundance. Regarding the fluxes of nutrients, both areas (with and without vegetation) showed under dark conditions an efflux of NH_4-N in all seasons, with the exception of vegetated sediment in spring. Under light incubations, NH_4-N was consumed in autumn (*S. maritima* colonized sediment and sediment without vegetation) and in summer (vegetated sediment) (Figure 2). Concerning NO_x-N, in *S. maritima* vegetated sediment, it was consumed under dark incubations in all seasons. Light incubations showed an efflux of NO_x-N in autumn, but consumption in winter. Spring and summer NO_x-N fluxes showed high variability, so it is not clear if there is efflux or consumption. There are no differences between vegetated sediment and bare bottom during these seasons. Bare bottom does not show a clear trend on NO_x-N fluxes (Figure 2).

Table 2. Average concentrations (± SE) of oxygen and nutrient (NH_4-N, NO_x-N) of the incubation water, in the initial conditions.

	Autumn	**Winter**	**Spring**	**Summer**
	Avrge (± SE)	Avrge (± SE)	Avrge (± SE)	Avrge (± SE)
O_2	219 (± 8)	221 (± 3)	210 (± 10)	194 (± 5)
NH_4-N	37.7 (± 0.2)	22.8 (± 3.2)	38.1 (± 8.7)	33.6 (± 7.6)
NO_x-N	46.6 (± 1.4)	43.2 (± 4.2)	34.7 (± 1.7)	25.6 (± 2.0)

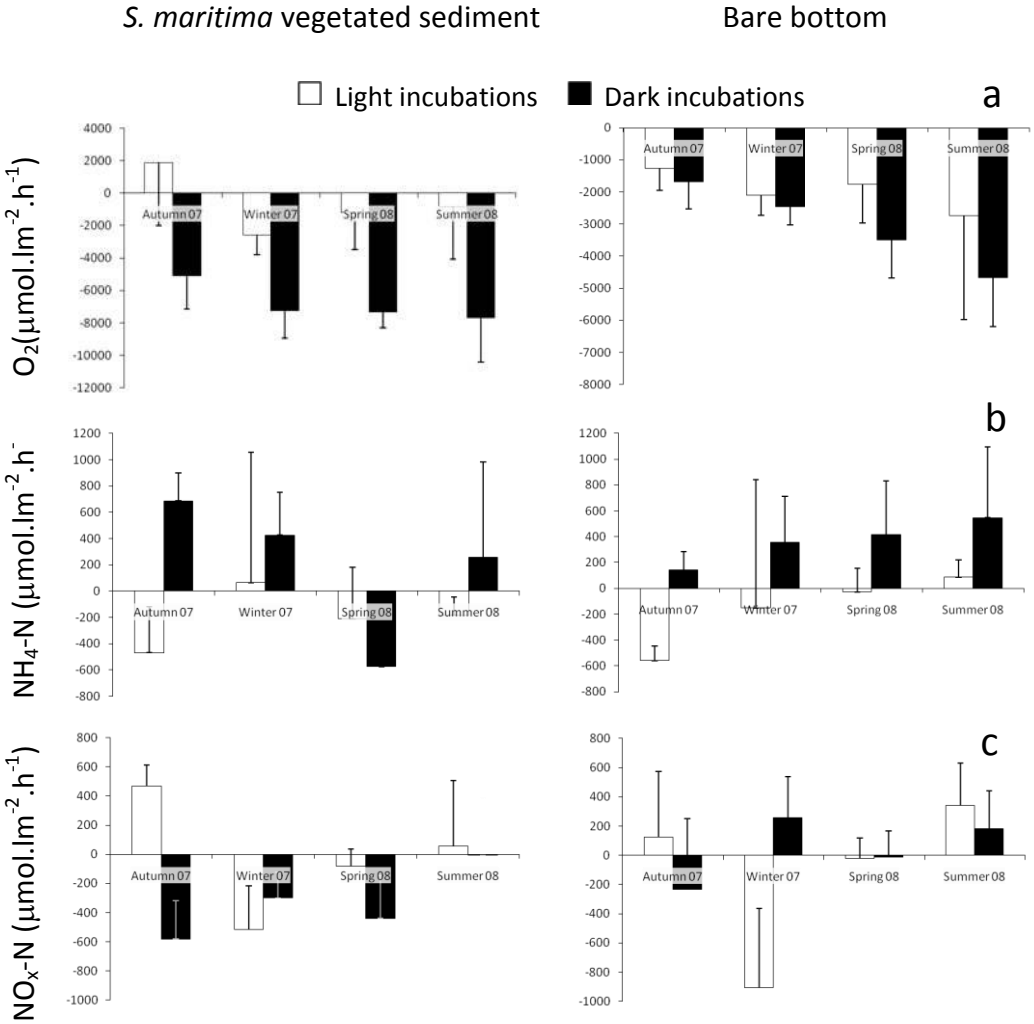


Figure 2. Oxygen and nutrient fluxes (avrg ± SD) in *S. maritima* vegetated sediment and in bare bottom along the year. Positive fluxes mean oxygen or nutrient efflux from the sediment, while negative fluxes represent uptake by the sediment. Light (white bars) and dark (black bars) incubation results are shown. (a – O_2 fluxes; b - NH_4-N fluxes; c - NO_x-N).

^{15}N- IPT assumptions

D_w (D^{15}; denitrification of bottom water NO_3^-) is significantly correlated to the $^{15}NO_3$ concentration in the water column ($p < 0.05$; $r = 0.9816$), while D_n (D^{14}; coupled nitrification-denitrification) is constant at all the $^{15}NO_3$ tested concentrations ($p > 0.05$; $r_s = 0.3907$) (Figure 3). The results suggest that all assumptions of IPT are fulfilled (Nielsen, 1992; Rysgaard et al., 1994; Steingruber et al., 2001; Eyre et al., 2002), which justifies the use of the IPT in this system.

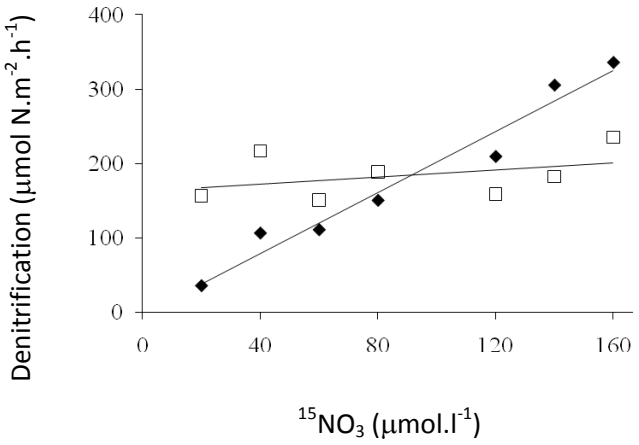


Figure 3. Denitrification (D_n and D_w) along an increasing $^{15}NO_3$ concentration series. D_w (black lozenges) is correlated with the $^{15}NO_3$ in the water column ($p < 0.05$; $r = 0.9816$), while coupled nitrification-denitrification (D_n; white squares) is similar with increasing $^{15}NO_3$ ($p > 0.05$; $r_s = 0.3907$).

Denitrification measurements

Within each season, denitrification rates between areas (with and without vegetation) are not significantly different, except in winter under dark conditions, in which the presence of *S. maritima* significantly enhanced denitrification rates (697 ± 497 µmol $N.m^{-2}.h^{-1}$) (Figure 4).

In autumn, values ranged between 51 ± 22 and 69 ± 7 µmol $N.m^{-2}.h^{-1}$ and there were no significant differences in total denitrification rates between dark and light incubations within each area, or between areas under the same incubation conditions (Figure 4A). In winter, total denitrification rate was significantly higher in the presence of *S. maritima* (Two-way ANOVA, $F = 24.06$, $p < 0.05$), and under dark conditions (Two-way ANOVA, $F = 5.48$, $p < 0.05$), but there was no interaction between these two variables (Figure 4A). In spring, values ranged between

80 ± 5 and 128 ± 61 μmol $N.m^{-2}.h^{-1}$, and there were no significant differences in total denitrification rates between both areas under the same incubation conditions, nor between dark and light incubations within each area. In addition, there were no differences in summer incubations between type of sediment (vegetated *vs* sediment without vegetation) and light *vs* dark conditions, and values ranged between 85 ± 36 and 151 ± 24 μmol $N.m^{-2}.h^{-1}$ (Figure 4A). Figure 4B shows that for bare bottom areas, denitrification rates were significantly higher under dark conditions in summer, while in colonized areas, denitrification rates were significantly higher in light and dark conditions in winter. In addition, between seasons and within each season, the contribution of D_n (coupled nitrification-denitrification) and D_w (denitrification of bottom-water NO_3^-) to denitrification rates between areas (with and without vegetation) do not show a clear trend (Table 3).

Discussion

Denitrification rates and nutrient flux measurements in this study were highly variable, similarly to other works (e.g. Cabrita and Brotas, 2000; Erikson et al., 2003; Poulin et al., 2007). This variability results from the natural variability within the system, given the number of parameters/variables that may interact and influence all these processes. These variables can be the abundance of biota (from microorganisms to macrofauna, and from MPB to macrophytes) that may have an interacting effect in these biogeochemical processes (e.g. Lillebø et al., 1999; Gilbert et al., 2003; Erickson et al., 2003; Risgaard-Petersen, 2003; Hou et al., 2007).

Potential nitrification allows estimating the nitrifier microbial community and preview D_n (Henrikson et al., 1981). In the Tagus estuary, potential nitrification rates were significantly higher in autumn and winter and there were no statistically significant differences between bare bottom and *S. maritima* vegetated sediment. Two main reasons may justify these seasonal differences: i) during the warmer grow season, plants and MPB may outcompete nitrifiers; ii) in this warm-temperate system, the water temperature in autumn/winter ranges between 15 and 17 °C, which is in accordance with a previous study performed at Tagus estuary (Gameiro et al., 2007). Actually, denitrification rates at Tagus estuary were, in winter, forty times higher than at a Canadian salt marsh (from 2 to 12 °C) (Poulin et al., 2007), which may be explained (within other factors) by higher temperature.

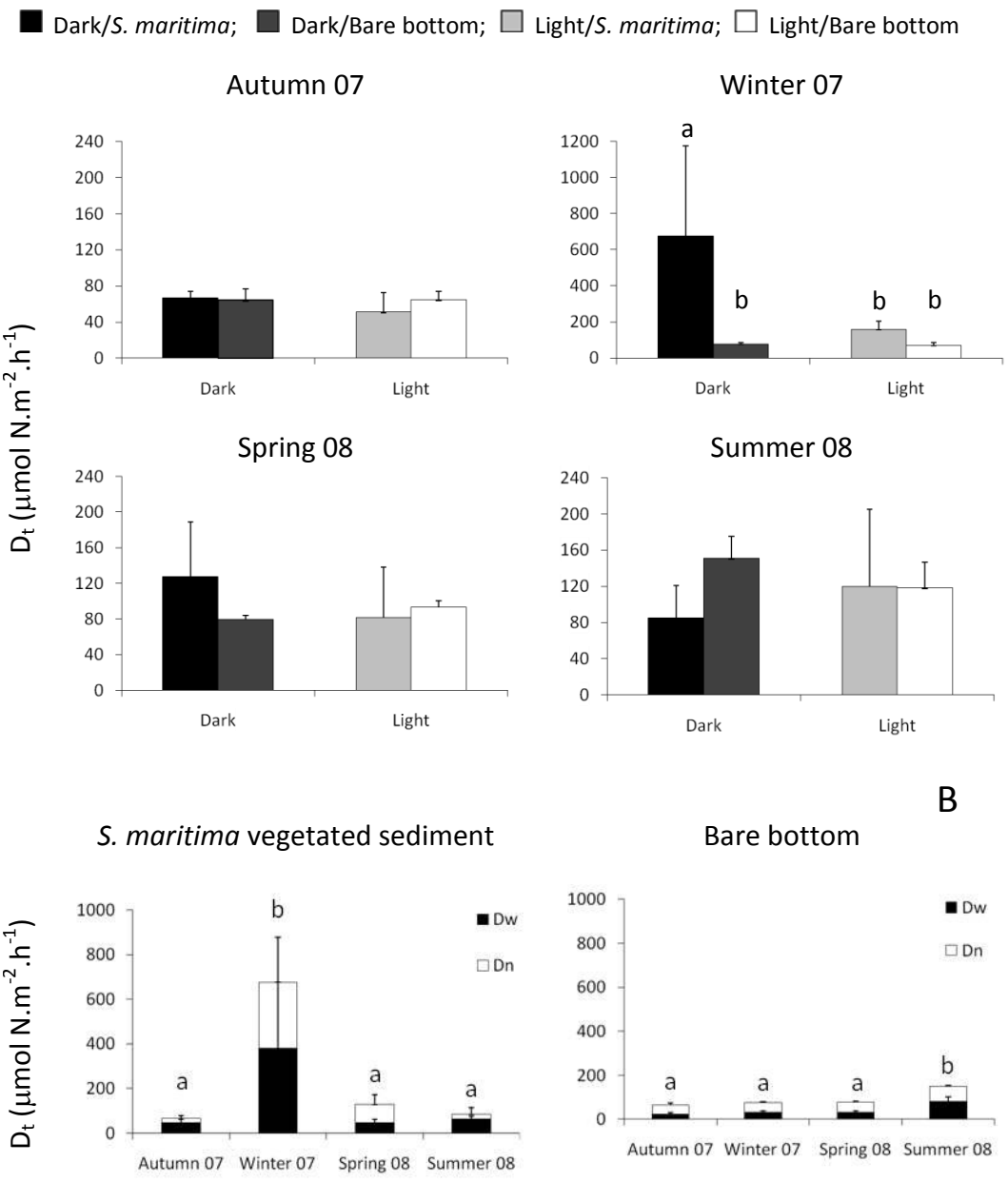


Figure 4. (A) Total denitrification (D_t) (avrg ± SD; n = 3 in autumn; n = 5 in the other seasons) in different seasons, comparing dark *vs* light conditions, and *S. maritima* vegetated sediment *vs* bare bottom. (B) D_t (avrg ± SD) in *Spartina* vegetated sediment and bare bottom in dark conditions. D_w (black bar) and D_n (white bar) absolute values are shown (µmol $N.m^{-2}.h^{-1}$; avrg ± SD; n = 3 in autumn; n = 5 in the other seasons). Different lowercase letters (a, b) means statistical significant differences ($p < 0.05$).

Table 3. Seasonal or annual denitrification in salt marshes: bare bottom and vegetated sediment ability to remove N in salt marshes through denitrification. All these studies were performed using ^{15}N-isotope pairing technique, and values shown include both light and dark incubations (single values correspond to the average, while ranges correspond to minimum and maximum values recorded).

Denitrification (μmol N.m^{-2}.h^{-1})		**Vegetated sediment**				**Bare bottom**				**Reference**
		Autumn	**Winter**	**Spring**	**Summer**	**Autumn**	**Winter**	**Spring**	**Summer**	
Tagus Estuary, Portugal	**D_t**	**51-67**	**158-676**	**82-128**	**85-120**	**64-65**	**71-76**	**80-94**	**119-151**	This study
	D_w	19-48	57-381	34-46	37-63	24-27	23-32	32-43	41-81	
	D_n	19-32	102-295	48-81	22-83	38-40	44-48	47-51	70-78	
Tagus Estuary, Portugal	**D_t**	**-**	**-**	**-**	**-**	**0-100 (R) 0-35 (P)**	**20-250 (R) 45-150 (P)**	**-**	**0-50 (R) 0-55 (P)**	Cabrita and Brotas, 2000[a]
	D_w	-	-	-	-	10-30 (R) 0-28 (P)	10-24(R) 0-38(P)	- -	0-10 (R) 0-13 (P)	
	D_n	-	-	-	-	0-65 (R) 0-10 (P)	5-200(R) 0-55 (P)	- -	0-12 (R) 0-40 (P)	
Venice lagoon, Italy	**Dt**	**125-250**	**-**	**21-125**	**7-21**	**143-286**	**-**	**72-143**	**14-43**	Erickson et al., 2003[b]
Colne Point, England	**D_t**	**8.3**				**-**	**-**	**-**	**-**	Aziz and Nedwell, 1986[c]
Great Sippewissett marsh, USA	**D_t**	**75**				**-**	**-**	**-**	**-**	White and Howes, 1994[d]
Pointe-au-Père, Canada	**Dt**	**11-25**	**6-15**	**-**	**18-42**	**-**	**-**	**-**	**-**	Poulin et al., 2007[e]
Kertinge Nor, Denmark	**D_t**	**-**	**-**	**-**	**-**	**3-5**	**35-75**	**20-30**	**7.5-12.5**	Rysgaard et al., 1995
	D_w	-	-	-	-	<1	10-16	10-15	<3	
	D_n	-	-	-	-	2-5	20-60	7.5-15	7.5-12.5	
Estuarine fjord, Denmark	**D_t**	**-**	**-**	**-**	**-**	**-**	**29**	**8-17**	**5**	Christensen et al., 2000

[a] R-Rosário salt marsh; P-Pancas salt marsh
[b] Data in bare bottom column corresponds to creek sediment; study performed with *Limonium serotinum*
[c] Study performed with *Puccinelia/Halimione* [e]
[d] Cape Code, MS,USA; study performed with *Spartina alterniflora*
[e] St. Lawrence estuary, Canada; study performed with *Spartina alterniflora*

It has been shown that *S. maritima* is able to control the efflux of ammonium (NH_4-N) during spring/summer, especially during the day (Lillebø et al., 2002), which is also in agreement with the results of the fluxes of this study. Concerning NO_x-N there was always a net uptake.

Total denitrification ranged between 64 ± 13 and 151 ± 24 μmol $N.m^{-2}.h^{-1}$ in sediment without vegetation, and between 51 ± 22 and 676 ± 497 μmol $N.m^{-2}.h^{-1}$ in *S. maritima* vegetated sediment. As a whole, denitrification is not higher in the marshes, considering seasons and dark/light conditions. However, under winter dark conditions rates were significantly higher in the vegetated sediment, meaning that in an annual basis areas colonized by *S. maritima* may enhance removal of nitrogen through denitrification. This seems to be related to the higher potential nitrification, as well as to the higher availability of inorganic nitrogen (namely NO_x) in the water column (Koch et al., 1992; Thompson et al., 1995; Ericksson et al., 2003) derived from freshwater inputs during winter. The greater availability of nitrate reduces the competition for nitrogen within the sediment and may contribute to a higher denitrification (Rysgaard et al., 1995; Ogilvie et al., 1997; Cabrita and Brotas, 2000). Moreover, there is less competition between plants/MPB and microbial community/nitrifying bacteria during winter, especially in dark conditions due to less NH_4-N uptake (Rysgaard et al., 1993, 1995; Risgaard-Petersen et al., 1994; Lillebø et al., 2002). In summer, although denitrification rates in bare bottom areas are in the same range as in the vegetated ones, the rates in bare bottom and dark conditions are significantly higher than in other seasons. Warmer water temperatures during summer contribute to an increase of denitrification, which may be explained by a lower competition for NH_4-N during dark conditions (less uptake of ammonium by MPB), as already described for *S. maritima*. Cabrita and Brotas (2000) did not find either significant differences in denitrification rates in sediments without vegetation under dark and light conditions.

Table 3 summarizes results of seasonal or annual denitrification rates in salt marshes (using ^{15}N-IPT). Compared to the selected studies, denitrification rates in bare bottom were within the same range as Venice lagoon (Erickson et al., 2003), as well as within the range recorded previously in the Tagus estuary (Cabrita and Brotas, 2000); but higher than those recorded in Denmark (Rysgaard et al., 1995; Christensen et al., 2000). On the other hand,

denitrification rates in Tagus areas colonized by *S. maritima* were comparatively higher than in other vegetated sediments, namely by *Limonium serotinum* in Venice lagoon (Erickson et al., 2003). The comparisons with other vegetated sediments, namely *Spartina alterniflora* (Valiela and Teal, 1979; White and Howes, 1994) and *Puccinelia/Halimione* (Aziz and Nedwell, 1986 in White and Howes, 1994), becomes limited due to differences in methodologies, even though all were based on tracing ^{15}N. Nevertheless, differences between the comparable results may be due to two main reasons: i) species specific interactions, namely with the microbial community and competition with nitrifiers; ii) geographical environmental characteristics, namely temperature range and seasonal availability of nitrate. More specifically, differences in denitrification rates between ecosystems may be due to several physical, chemical and biological factors such as temperature, light, NO_3 concentrations, oxygen availability, benthic microalgae, benthic fauna, presence/absence of plants, among others (e.g. Kaplan et al., 1979; Valiela and Teal, 1979; Risgaard-Petersen et al., 1994; Cornwell et al., 1999; Herbert, 1999; Pinã-Ochoa and Alvares-Cobelas, 2006; Erickson et al., 2003; Poulin et al., 2007; Koop-Jacobsen and Giblin, 2009).

The relative contribution of D_n and D_w to total denitrification in sediments without vegetation is similar, which is in accordance with Cabrita and Brotas (2000). This may be due to two main reasons: i) in winter the potential nitrification is higher (which may be reflected in D_n); ii) this also corresponds to the rainy period in which the nitrate concentration in the water column is higher (which may be reflected in D_w). In sediments colonized by *S. maritima* the relative contribution of D_n and D_w to total denitrification is more variable, although there is not a seasonal variation as well. Nitrification process is generally limited by low oxygen and ammonium concentrations (Henriksen and Kemp, 1988). D_n depends on oxygen penetration and ammonium availability, which may be more variable at plants rhizosediments. In addition, oxygen penetration depends on plants and microbial activity and infauna bioturbation, whilst ammonium availability depends on the balance between ammonification and processes using NH_4^+ (e.g. uptake by primary producers and nitrification).

Conclusions

As in other studies denitrification is influenced by multiple interacting variables, resulting in inherent variability under identical experimental conditions. When compared to sediments without vegetation, denitrification rates in *S. maritima* salt marshes, was significantly higher in winter, potentially contributing to a higher reduction of the land driven

nitrogen inputs to the Tagus estuary at this time of the year. This corresponds to the period when nitrate concentrations were higher due to higher freshwater runoff and the temperature range was 15 - 17 °C. These two conditions may favor potential nitrification, which was also higher in winter. Additionally, at this time of the year, competition between primary producers (*S. maritima* and MPB) and nitrifiers is lower. Although there are still uncertainties concerning the fate of all land-derived nitrogen (Galloway et al., 2004), denitrification seems to be an important reactive nitrogen sink, meaning that even in heavily altered regions, rivers, although important sources of nitrogen to coastal systems, represent small sources of reactive nitrogen to the open ocean (Galloway et al., 2008). Our study shows that *S. maritima* marshes contribute to the reduction of the land-driven nitrogen loading to the open ocean. However, on an annual basis it cannot be stated that it is significantly different from the sediments without vegetation. Nevertheless, the significantly higher contribution of *S. martima* marshes in N removal during winter, contributes to the reduction of nitrate availability in the following spring.

References

Bendschneider, K., Robinson, R.J., 1952. A new spectrophotometric method for the determination of nitrite in sea water. Journal of Marine Research 11(1), 87-96.

Bouchard, V., Lefeuvre, J.C., 2000. Primary production and macro-detritus dynamics in a European salt marsh: carbon and nitrogen budgets. Aquatic Botany 67, 23-42.

Cabrita, M.T., Brotas, V., 2000. Seasonal variation in denitrification and dissolved nitrogen fluxes in intertidal sediments of the Tagus estuary. Marine Ecology Progress Series 202, 51–65.

Caçador, I., Costa, A.L., Vale, C., 2007. Nitrogen sequestration capacity of two salt marshes from the Tagus estuary. Hydrobiologia 587, 137-145.

Christensen, P.B., Rysgaard, S., Sloth, N.P., Dalsgaard, T., Schwaerter, S., 2000. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology 21, 73–84.

Cornwell, J.C., Kemp, W.M., Kana, T.M., 1999. Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquatic Biology 33, 41–54.

Dalsgaard, T., Nielsen, L.P., Brotas, V., Viaroli, P., Underwood, G., Nedwell, D.B., Sundbäck, K., Rysgaard, S., Miles, A., Bartoli, M., Dong, L., Thornton, D.C.O., Ottosen, L.D.M., Castaldelli, G., Risgaard-Petersen, N., 2000. Protocol handbook for NICE - Nitrogen Cycling in Estuaries: A project under the EU research programme: Marine Science and Technology (MAST III). National Environmental Research Institute, Silkeborg, Denmark.

Dalsgaard, T., Thamdrup, B., Canfield, D.E. 2005. Anaerobic ammonium oxidation (anammox) in the marine environment, Research in Microbiology 156, 457-464.

Edwards, K.R., Mills, K.P., 2005. Aboveground and belowground productivity of *Spartina arteniflora* (smooth cordgrass) in natural and created Louisiana salt marshes. Estuaries 28, 252-265.

Eriksson, P.G., Svensson, J.M., Carrer, G.M., 2003. Temporal changes and spatial variation of soil oxygen consumption, nitrification and denitrification rates in a tidal salt marsh of the Lagoon of Venice, Italy. Estuarine, Coastal and Shelf Science 58, 861-871.

Eyre, B.D., Rysgaard, S., Dalsgaard, T., Christensen, P.B., 2002. Comparison of isotope pairing and N_2 : Ar methods for measuring sediment-denitrification-assumptions, modifications, and implications. Estuaries 25(6A), 1077-1087.

Galloway, J.N., 1998. The global nitrogen cycle: changes and consequences. Environmental Pollution 102, 15-24.

Galloway, J.N., Townsend A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: Recent trends, questions, and potential solutions. Science 320, 889–892.

Gameiro, C., Cartaxana, P., Brotas, V., 2007. Environmental drivers of phytoplankton distribution and composition in Tagus Estuary, Portugal. Estuarine, Coastal and Shelf Science 75(1/2), 21-34.

Gilbert, F., Stora, G., Bonin, P., 1998. Influence of bioturbation on denitrification activity in Mediterranean coastal sediments: an in situ experimental approach. Marine Ecology Progress Series 163, 99-107.

Henriksen, K., Hansen, J.I., Blackburn, T.H., 1981. Rates of nitrification, distribution of nitrifying bacteria, and nitrate fluxes in different types of sediment from Danish waters. Marine Biology 61, 299-304.

Henriksen, K., Kemp, W.M., 1988. Nitrification in estuarine and coastal marine sediments: methods, patterns and regulating factors. In: Blackburn, T. H., Sorensen, J. (ed.) Nitrogen cycling in coastal marine environments. John Wiley and sons, New York, p. 207-250.

Herbert, R.A., 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiol Review 23, 563-590.

Hou, L.J., Liu, M., Xu, S.Y., Ou, D.N., Yu, J., Cheng, S.B., Lin, X., Yang, Y. 2007. The effects of semi-lunar spring and neap tidal change on nitrification, denitrification and N_2O vertical distribution in the intertidal sediments of the Yangtze estuary, China. Estuarine, Coastal and Shelf Science 73, 607-616.

Howarth, R.W., Billen, G., Swaney, D., Townsend, A., Jarworski, N., Lajtha, K.,Downing, J.A., Elmgren, R., Caraco, N., Jordan, T., Berendse, F., Freney, F.,Kueyarov, V., Murdoch, P., Zhu, Z.-L., 1996. Riverine inputs of nitrogen to the North Atlantic Ocean: fluxes and human influences. Biogeochemistry 35, 75–139.

Hulth, S., Aller, R.C., Canfield, D.E., Dalsgaard, T., Engström, P., Gilbert, F., Sundbäck, K., Thamdrup, B., 2005. Nitrogen removal in marine environments: recent findings and future research challenges. Marine Chemistry 94, 125-145.

Jaffe, D.A., 2000. The nitrogen cycle, p. 322–342. *In* M. C. Jacobson, R. J. Charlson, H. Rodhe, and G. H. Orians (ed), Earth system science. Academic Press, San Diego, Calif.

Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochemie und Physiologie der Pflanzen 167, 191–194.

Kaplan, W., Valiela, I., Teal, J.M., 1979. Denitrification in salt marsh ecosystem. Limnology and Oceanography 24, 726–734.

Koch, M.S., Malby, E., Oliver, G.A., Bakker, S.A., 1992. Factor controlling denitrification rates of tidal mudflats and fringing salt marshes in south-west England. Estuarine, Coastal and Shelf Science 34, 471–485.

Koop-Jakobsen, K., Giblin, A.E., 2009. Anammox in Tidal Marsh Sediments: The Role of Salinity, Nitrogen Loading, and Marsh Vegetation. Estuaries and Coasts 32, 238–245.

Koroleff, F., 1969/1970. Direct determination of ammonia in natural waters as indophenol blue. Int Counc Explor Sea (ICES) Comm Meet Pap 1969/C:9; revised 1970, 19–22.

Lee, R.W., Kraus, D.W., Doeller, J.E., 1999 Oxidation of sulfide by *Spartina alterniflora* roots. Limnology and Oceanography 44(4), 1155–1159.

Lillebø, A.I., Flindt, M.R., Pardal, M.A., Marques, J.C., 1999. Population structure, dynamics and production of Hydrobia ulvae (Pennant) (Mollusca: Prosobranchia) along an eutrophication gradient in the Mondego estuary (Portugal). Acta Oecologica 20(4), 289-304.

Lillebø, A.I., Flindt, M.R., Pardal, M.A., Martins, I., Neto, J.M., Marques, J.C., 2002. Nutrient dynamics in the intertidal pools of the Mondego estuary. IIdSeasonal efflux of PO_4-P and NH_4-N in bare bottom and vegetated pools. In: Pardal, M.A., Marques, J.C., Graça, M.A. (eds) Aquatic Ecology of the Mondego River Basin. Global Importance of Local Experience. Imprensa da Universidade de Coimbra, Coimbra, p 257-272.

Lorenzen, C.J., 1967. Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography 12, 343–346.

Madureira, M.J., Vale, C., Simões Gonçalves, M.L., 1997. Effect of plants on sulphur geochemistry in the Tagus salt-marshes sediments. Marine Chemistry 58, 27-37.

Maricle, B.R., Lee, R.W., 2002. Aerenchyma development and oxygen transport in the estuarine cordgrasses *Spartina arteniflora* and *S. anglica*. Aquatic Botany 74, 109-120.

Nielsen, L.P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiology Ecology 86, 357-362.

Ogilvie, B., Nedwell, D.B., Harrison, R.M., Robinson, A., Sage, A., 1997. High nitrate, muddy estuaries as nitrogen sinks: the nitrogen budget of the River Colne estuary (United Kingdom). Marine Ecology Progress Series 150, 217–228.

Piña-Ochoa, E., Álvarez-Cobelas, M., 2006. Denitrification in aquatic environments: a cross-system analysis. Biogeochemistry 81, 111-130.

Poulin, P., Pelletier, E., Saint-Louis, R., 2007. Seasonal variability of denitrification efficiency in northern salt marshes: An example from the St. Lawrence Estuary. Marine Environmental Research 63, 490–505.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh capacity retention for heavy metals. Environmental Pollution 146, 147-154.

Reddy, K.R., Patrick, W.H., Lindau, C.H., 1989. Nitrification-denitrification at the plant root-sediment interface in wetlands. Limnology and Oceanography 34, 1004-1013.

Risgaard-Petersen, N., 2003. Coupled nitrification-denitrification in autotrophic and heterotrophic estuarine sediment: on the influence of benthic microalgae. Limnology and Oceanography 48, 93–105.

Risgaard-Petersen, N., Rysgaard, S., Nielsen, L.P., Revsbech, N.P., 1994. Diurnal variation of denitrification and nitrification in sediments colonized by benthic microphytes. Limnology and Oceanography 39(3), 573–579.

Rysgaard, S., Christensen, P.B., Nielsen, L.P., 1995. Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. Marine Ecology Progress Series 126, 111–121.

Rysgaard, S., Risgaard-Petersen, N., Nielsen, L.P., Revsbech, N.P., 1993. Nitrification and Denitrification in Lake and Estuarine Sediments Measured by the ^{15}N Dilution Technique and Isotope Pairing. Applied Environmental Microbiology 59(7), 2093-2098.

Schlesinger, W.H., 1997. Biogeochemistry, 2nd ed. Academic Press, San Diego, Calif.

Seitzinger, S.P., 1988. Denitrification in fresh and coastal marine ecosystems: ecological and geochemical significance. Limnology and Oceanography 33, 702–724.

Silvennoinen, H., Liikanen, A., Torssonen, J., Stange, C.F., Martikainen, P.J., 2008. Denitrification and N2O effluxes in the Bothnian Bay (northern Baltic Sea) river sediments as affected by temperature under different oxygen concentrations. Biogeochemistry 88, 63-72

Simas, T., Nunes, J.P., Ferreira, J.G., 2001. Effects of global climate change on coastal salt marshes. Ecological Modelling 139, 1-5.

Sousa, A.I., Lillebø, A.I., Caçador, I., Pardal, M.A., 2008. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628–635.

Steingruber, S.M., Friedrich, J., Gachter, R., Wehrli, B., 2001. Measurement of denitrification in sediments with the ^{15}N isotope pairing technique. Applied Environmental Microbiology 67, 3771–3778.

Sundbäck, K., Miles, A., Linares, F., 2006. Nitrogen dynamics in nontidal littoral sediments: Role of microphytobenthos and denitrification. Estuaries and Coasts 29(6), 1196-1211.

Teal, J.M., Howes, B.L., 2000. Salt marsh values: retrospection from the end of the century. In: Weinstein, M. P. and D. A. Kreeger. Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishing. Dordrecht, the Netherlands, p 9-22.

Thompson, S.P., 1995. Seasonal pattern of nitrification and denitrification in a natural and a restored salt marsh. Estuaries 18, 399–408.

Trimmer, M., Gowen, R.J., Stewart, B.M., 2003. Changes in sediment processes across the western Irish Sea front. Estuarine, Coastal and Shelf Science 56, 1011-1019.

Valiela, I., Cole, M.L., 2002. Comparative evidence that salt marshes and mangroves may protect seagrass meadows from land-derived nitrogen loads. Ecosystems 5, 92–102.

Valiela, I., Teal, J.M., 1979. The nitrogen budget of a salt marsh ecosystem. Nature 280, 652-656.

White, D.S., Howes, B.L., 1994. Long-term ^{15}N-nitrogen retention in the vegetated sediments of a New England salt marsh. Limnology and Oceanography 39(8), 1878-1892.

CHAPTER II

Metals Contamination In Salt Marshes

CHAPTER II – Metals contamination in salt marshes

Introduction

Parallel to the increase of global population, estuaries have been subjected to different pollutants: organic (e.g. nutrients, herbicides, insecticides) and inorganic compounds, namely metals, combustible substances, hazardous wastes (e.g. DDT, PCB - polychlorinated biphenyls), petroleum products (e.g. PAH - polycyclic aromatic hydrocarbons). These contaminants can persist for decades in these ecosystems within sediments affecting both flora and fauna. Particularly, long-term exposures to residual petroleum have impacts on *S. alterniflora* biomass and coastal erosion leading to the decline of marsh habitat (Culbertson et al., 2008).

The degradation effects of spilled oil on marine ecosystems, namely on salt marshes, is well documented (e.g. Burns and Teal, 1971; Sanders et al., 1980; Teal et al., 1992). In addition, salt marsh plants' contribution to ameliorate metals or other pollutants contamination in estuarine ecosystems is widely recognized (Boorman, 2003; Windham et al., 2003; Almeida et al., 2004; Weis and Weis, 2004; Hwang et al., 2006; Quan et al., 2007; Reboreda and Caçador, 2007; Almeida et al., 2008; Suntornvongsagul et al., 2007, Du Laing et al., 2009; Caçador et al., 2009). This sink function can occur through different phytoremediation processes, namely phytoextraction, which will be the focus of the present chapter.

Phytoremediation

Phytoremediation, or plant-assisted bioremediation, is defined by the use of green or higher terrestrial plants to remove pollutants from the environment or to render them harmless (Salt et al., 1995; Wenzel, 2009). This emergent technology combines interdisciplinary research approaches, it is considered environmentally friendly, cost-effective and can be used to remove or degrade both organic and inorganic pollutants present in different substrates (air, liquid - e.g. water, or solid – e.g. soil/sediment) (Salt et al., 1998). Phytoremediation can be divided in different areas (Salt et al., 1998; Gosh and Singh, 2005; Wenzel , 2009; Jabeen et al., 2009; Dhir et al., 2009), depending on the process involved:

- Phytoextraction: consists in the ability of the plant to remove metals or organic pollutants from the soil through its uptake by the roots, and subsequent store/hyper-accumulation in its shoots/biomass;

- Phytodegradation: the ability of plants, through metabolic processes, and microorganisms from the rhizosphere to degrade organic pollutants;
- Rhizofiltration: the ability of plants to absorb and adsorb pollutants, mainly metals but also excess nutrients, from the water and waste-water streams;
- Phytostabilization (and immobilization): plants' capacity to reduce the bioavailability of pollutants in the environment by mechanically stabilizing the site and reducing pollutant transfer to other ecosystem compartments and the food chain;
- Phytovolatilization/rhizovolatilization: the ability of plants to remove pollutants from the soil/sediment by transforming them in volatile compounds which are released to the atmosphere

References

Almeida, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D., 2004. Influence of the sea rush *Juncus maritimus* on metal concentration and speciation in estuarine sediment colonized by the plant. Environmental Science and Technology 38, 3112–8.

Boorman, L.A., 2003. Saltmarsh Review. An overview of coastal saltmarshes, their dynamic and sensitivity characteristics for conservation and management. JNCC, Peterborough. On-line version at http://www.jncc.gov.uk/pdf/jncc334.pdf Accessed 14 Oct 2009

Burns, K.A., Teal, J.M., 1971. Hydrocarbon Incorporation into the Salt Marsh Ecosystem after the West Falmouth Oil Spill. Technical Report 71–69. Almeida, C.M.R., Mucha, A.P., Bordalo, A.A., Vasconcelos, M.T.S.D., 2008. Influence of a salt marsh plant (*Halimione portulacoides*) on the concentrations and potential mobility of metals in sediments. Science of the Total Environment 403, 188–195.

Culbertson, J.B., Valiela, I.,Pickart, M., Peacock, E.E., Reddy, C.M., 2008. Long-term consequences of residual petroleum on salt marsh grass. Journal of Applied Ecology 45, 1284–1292.

Dhir, B., Sharmila, P., Saradhi, P.P., 2009. Potential of Aquatic Macrophytes for Removing Contaminants from the Environment. Critical Reviews in Environmental Science and Technology 39, 754–781.

Du Laing, G., Rinklebe, J., Vandecasteele, B., Meers, E., Tack, F.M.G., 2009. Trace metal behaviour in estuarine and riverine floodplain soils and sediments: A review. Science of the Total Environment 407, 3972-3985.

Ghosh, M., Singh, S.P., 2005. A review on phytoremediation of heavy metals and utilization of its byproducts. Applied Ecology and Environmental Research 3(1), 1-18.

Jabeen, R., Ahmad, A., Iqbal, M., 2009. Phytoremediation of Heavy Metals: Physiological and Molecular Mechanisms. Botanical Review 75, 339-364.

Quan W.M., Han, J.D., Shen, A.L., Ping, X.Y., Qian, P.L., Li, C.J., Shi, L.Y., Chen, Y.Q., 2007. Uptake and distribution of N, P and heavy metals in three dominant salt marsh macrophytes from Yangtze River estuary, China. Marine Environmental Research 64, 21-37.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh capacity retention for heavy metals. Environmental Pollution 146, 147-154.

Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I et al (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13:468–474.

Sanders, H.L., Grassle, J.F., Hampson, G.R., Morse, L.S., Garner-Price, S. Jones, C.C. (1980) Anatomy of an oil spill: long-term effects from the grounding of the barge Florida off West Falmouth. Massachusetts. Journal of Marine Research, 38, 265–380.

Suntornvongsagul, K., Burke, D.J., Hamerlynck, E.P., Hahn, D., 2007. Fate and effects of heavy metals in salt marsh sediments 149, 79-91.

Teal, J.M., Farrington, J.W., Burns, K.A., Stegeman, J.J., Tripp, B.W., Woodin,B., Phinney, C., 1992. The West Falmouth oil spill after 20 years: fate of fuel oil compounds and effects on animals. Marine Pollution Bulletin 24, 607–614.

Weis, J.S., Weis, P., 2004. Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. Environmental International 30, 685-700.

Wenzel, W.W., 2009. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321(1-2) 385-408.

Windham, L., Weis, J.S., Weis, P., 2003. Uptake and distribution of metals in two dominant salt marsh macrophytes, Spartina alterniflora (cordgrass) and Phragmites australis (common reed). Estuarine, Coastal and Shelf Science 56, 63–72.

Case study

1. Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites

Abstract

Salt marsh sediments can largely retain heavy metals, as well as salt marsh plants which are known by the capacity to sequestrate and inherently tolerate high metal concentrations. This work intended to understand the *Halimione portulacoides* (L.) Aellen strategies to prevent metal toxicity, showing the metal location in different plant organs and in the cell. This species is a common macrophyte in the Tagus estuary (Portugal). It was performed a sequential extraction on leave, stem and root of *H. portulacoides* in order to determine and compare the metal (Zn, Pb, Co, Cd, Ni and Cu) concentration in several fractions of the plant material (ethanolic, aqueous, proteic, pectic, polissacaridic, lenhinic and cellulosic). This study shows that all organs of *H. portulacoides* mostly retain metals in the cell wall (65% is the average for all studied metals stored in roots' cell wall, 55% in stems and 53% in leaves) and the metal content in the intracellular compartment is much lower (21% in roots, 25% in stems and 32% in leaves). Therefore, the high metal levels in the sedimentary environment do not causes toxicity to salt marsh plants because plant immobilizes them in different cell compartments (cell wall + proteic fraction + intracellular), outside key metabolic sites, which may be crucial to the surviving of *H. portulacoides* in highly metal contaminated salt marshes.

Keywords: Compartmentation; *Halimione portulacoides*; Heavy metals; Phytotoxicity; Sequential extraction

Introduction

Estuarine salt marshes are frequently highly contaminated with metals, due to human and industrial activities occurring in the estuaries and adjacent areas. However, these contaminants must be in an available form so they can be up taken by salt marsh plants (Greger, 2004), which are known to tolerate and accumulate high levels of heavy metals. It seems there is an innate tolerance to metals in wetland plants (McCabe et al., 2001), eventually explained by the biogeochemistry of the rhizosphere (Otte et al., 2004 in Matthews

et al., 2004). The solubility and availability of metals for plants may be affected by several factors such as their loading rate, chemical characteristics, pH, redox potential, soil texture, clay content and organic matter content, cation exchange capacity, etc. (Greger, 2004), which determines the different uptake by different plant species and at different locations. Salt marsh plants have the ability to uptake metals and then they are translocated within the plant, modifying the metal concentration in different organs. These plants generally accumulate different percentage of metals in the below- and aboveground parts, with higher percentage of metals in the roots rather than in aboveground part (Fitzgerald et al., 2003; see Matthews et al., 2004). Metal translocation can occur in the phloem, via the apoplast, and via the xylem, acropetally (Greger, 1999). Therefore, metal translocation and storage capacity differs with plant species and with metal (e.g. Stoltz and Greger, 2002).

In order to survive in metal contaminated salt marshes, salt marsh plants may have mechanisms to regulate (and distribute) internal and cell wall metal concentrations, according to their tolerance capacity, which determines their survival. Metal tolerance by plants and heavy metal detoxification may be achieved through metal complexation with ligands such as organic acids, amino acids and some members of mugineic acids which exist in plant tissues, and also by compartmentation (Hall, 2002; see Carrier et al., 2003 and references therein). So, metals can be stored/accumulated either in cell walls (e.g. Lozano-Rodriguez et al., 1997; Carrier et al., 2003), cytoplasm or in cell vacuoles (e.g. for Cd see Carrier et al., 2003). In order to maximize their detoxification and/or transport, plants control both the oxidation state and coordination environment of specific metallic elements (Salt et al., 2002). Direct coordination of the element (e.g. cadmium, nickel and zinc) by the plant, through the most chemically appropriate ligand leads to stable non-toxic complexes, and this is the mechanism used for detoxification of metals and metalloids (Salt et al., 2002).

The Tagus estuary is warm-temperate and is located near a highly populated and industrialized city (Lisbon) (Figure 1, chapter I, case study 2). Its salt marshes are colonized by several macrophyte species, namely *Halimione portulacoides* (L.) Aellen. These salt marshes retain heavy metals in their sediments, which are largely sequestrated and tolerated, by these macrophytes (Caçador et al., 2000; Reboreda et al., 2006). However, the toleration mechanism is not yet completely understood, not even the exact location of metal accumulation in the plants and cells. This work intends to study the metal compartmentation and location within the plant cell and in different organs of *Halimione portulacoides*. Do all plant organs (leaves, stems and roots) have the same capacity for retaining/sequestrate different heavy metals? Where are they accumulated: in the cell wall or intracellularly? It is scarce the knowledge

about heavy metal storage/location within the cells of *Halimione portulacoides*, and this work intends to focus on these aspects. Zinc, lead, cobalt, cadmium, nickel and copper were the analyzed heavy metals.

Materials and Methods

Sampling strategy and laboratorial processing

Samples of *Halimione portulacoides* plants were collected from monotypic stands in Rosário salt marsh, in the Tagus estuary (see Figure 1, chapter I, case study 2, page 56). It was sampled three squares of 0.3x0.3m^2, wherein the aboveground material was collected through harvesting and belowground material was collected by through sediment cores exactly on the same area. Afterwards, the samples were brought to the laboratory and were processed. *Halimione portulacoides* (above and belowground material) was rinsed with demineralised water, and dried during 48h (until constant weight) at 60 °C. Leaves, stems and belowground material were separated.

Heavy metal extraction procedure

It was performed a sequential extraction (adapted from Farago and Pitt, 1977), in order to know the metal partition in cellular constituents of *H. portulacoides*. Vegetal material from different plant organs (1 g DW; n=3), previously reduced to a powder, was processed individually in a soxhlet and the extraction solvents used were ethanol (80%), demineralised water, enzymatic solutions (pronase, cloranphenicol and pectinase), NaOH (0.5M) and HCl (5%). Lastly, an acid digestion was performed with HNO_3/$HClO_4$ (7:1, v:v) for plant samples and then put into the oven at 110 °C for 3h. After cooling, all extracts (ethanolic, aqueous, proteic, pectic, polissacaridic, lenhinic and cellulosic) were filtered through Whatman 42 filters (pore Ø 2.5 µm) and diluted until 10 ml, with demineralised water.

Metals bound to pectic, polissacaridic, lenhinic and cellulosic fractions are those bound to the cell wall, since these are constituents of the cell wall. The different types of proteins can not be determined using this extraction method which implies that its exact location in the cell can not be defined. The metals bound to some amino acids, chlorophyll, low weight compounds (all extracted by ethanol) and those extracted in the aqueous fraction were designated soluble metal (Farago and Pitt, 1977).

Analytical procedures

Metal concentrations in the *Halimione portulacoides* samples were determined by atomic absorption spectrometry (Perkin Elmer A Analyst 100). International certificate standard additions and sludge reference materials were used (Olea europeae BCR62).

Statistical analyses and calculations

Two-way ANOVA (analysis of variance) was performed for each metal to test for differences in metal concentration between plant organs (three levels) and extracted fractions (seven levels). Dixon's test was performed to detect outliers. Data were log-, log (x+1)- or 1/(x+0.5)- transformed when necessary, to achieve the homogeneity of variances (Cochran's Q test). Normality of the data was also assured (Kolmogorov-Smirnov test). Post-hoc comparisons was performed by Newman-Keuls test at α=0.05 significance level. Analyses were performed with STATISTICA 7.0 software package.

The translocation factor (TF) was calculated by the ratio of $[metal]_{aboveground}/[metal]_{root}$ and expressed the metals translocation within the plant, from roots to the aboveground part (Deng et al., 2004). It was also calculated the TF from roots to leaves and to stems, separately.

Results

Total metal concentrations (sum of metals from all extracted fractions) from different organs (roots, stems and leaves) of *Halimione portulacoides* show a common pattern: Zn > Pb > Cu > Ni > Co > Cd, ranging between 290.89 µg. g^{-1} DW of Zn in roots to 5.10 µg. g^{-1} DW of Cd in leaves (Table 1 and Figure 1). Zn presents five to 27 times higher concentration than the other metals, both in roots and aboveground material.

Roots present significantly higher metal concentrations than stems and leaves, for all studied metals (Two way ANOVA, $p<0.001$; Newman-Keuls test for Post-Hoc) (Figure 1).

Cd was the only metal wherein metal concentration in leaves was significantly lower than in stems, with all other metals presenting statistically the same concentrations in leaves and stems. The translocation of metals from the roots to the aboveground material can be expressed by the translocation factor (TF), and varied from 0.73 (Cu) to 1.11 (Cd) (Table 2). Instead, if we consider the TF for metals from roots to leaves, it varies from 0.33 for Cu to 0.47 for Zn. The TF range from roots to stems is from 0.40 (Cu) to 0.64 (Cd). Cd is the metal with the highest TFs and Cu presents the lowest ones.

Table 1. Metal concentrations (µg. g^{-1} DW) (average ± SD; n=3) on different fractions of *Halimione portulacoides* leaves, stems and roots, corresponding to extra- and intra-cellular location.

Plant organ	Fraction	Metal (µg. g^{-1} DW) (average±SD)					
		Zn	**Pb**	**Co**	**Cd**	**Ni**	**Cu**
Roots	**Ethanolic**	28.34 ±11.84	7.27 ±1.07	1.55 ±0.46	0.97 ±0.05	2.84 ±0.63	1.83 ±1.53
	Aquosous	33.37 ±26.01	10.27 ±2.09	2.01 ±0.35	1.14 ±0.13	3.11 ±0.90	2.74 ±1.68
	Proteic	77.70 ±35.27	5.30 ±1.81	1.56 ±0.73	1.15 ±0.54	2.12 ±1.09	7.70 ±3.61
	Pectic	53.40 ±32.27	8.29 ±2.59	1.76 ±0.49	1.26 ±0.52	2.27 ±0.65	9.89 ±7.52
	Polissacaridic	19.44 ±7.31	16.40 ±7.75	9.58 ±4.90	5.12 ±2.66	11.95 ±5.38	6.46 ±4.00
	Lignin	66.30 ±12.35	7.57 ±1.40	1.57 ±0.50	1.30 ±0.26	2.07 ±0.67	6.43 ±3.57
	Celulosis	12.33 ±9.01	0.01 ±0.007	1.47 ±1.25	0.04 ±0.07	2.30 ±0.00	1.55 ±0.58
	Total	290.89	55.13	19.50	10.99	26.65	36.60
Stems	**Ethanolic**	13.82 ±3.42	5.23 ±3.10	1.19 ±0.67	0.89 ±0.45	1.73 ±0.71	1.92 ±0.31
	Aquosous	15.93±11.21	4.99 ±2.40	1.18 ±0.60	0.85 ±0.44	1.97 ±0.43	1.79 ±0.98
	Proteic	78.90 ±44.55	4.45 ±1.72	1.01 ±0.17	0.81 ±0.34	1.48 ±0.47	3.66 ±1.62
	Pectic	15.73 ±4.43	4.13 ±1.49	0.77 ±0.23	0.63 ±0.18	1.31 ±0.37	1.56 ±1.64
	Polissacaridic	11.70 ±5.98	8.61 ±3.24	4.31 ±1.80	2.69 ±0.90	5.30 ±4.26	3.38 ±0.95
	Lignin	26.43 ±9.46	4.63 ±2.27	0.93 ±0.61	0.89 ±0.60	1.50 ±0.76	1.59 ±1.29
	Celulosis	3.68 ±1.86	0.00 ±0.00	0.06 ±0.10	0.29 ±0.36	0.87 ±0.55	0.70 ±0.36
	Total	166.19	32.04	9.47	7.04	14.14	14.60
Leaves	**Ethanolic**	13.63 ±5.65	5.27 ±1.02	1.85 ±0.60	1.15 ±0.33	2.00 ±0.55	3.08 ±1.85
	Aquosous	9.32 ±0.59	3.98 ±1.23	0.94 ±0.33	0.65 ±0.23	1.22 ±0.44	0.97 ±0.43
	Proteic	46.15±24.16	3.49 ±0.007	0.71 ±0.17	0.54 ±0.13	1.00 ±0.24	1.57 ±0.47
	Pectic	14.89±5.32	2.80 ±0.34	0.60 ±0.08	0.44 ±0.07	0.75 ±0.001	2.29 ±2.06
	Polissacaridic	27.79±34.27	2.56 ±2.75	2.78 ±2.71	1.69 ±1.53	3.95 ±3.03	2.61 ±2.00
	Lignin	16.62±13.44	3.38 ±0.71	0.73 ±0.21	0.54 ±0.16	0.99 ±0.27	0.81 ±0.26
	Celulosis	8.63±6.30	0.51 ±0.46	0.36 ±0.29	0.10 ±0.02	1.27 ±1.68	0.73 ±0.24
	Total	137.03	21.99	7.96	5.10	11.18	12.05

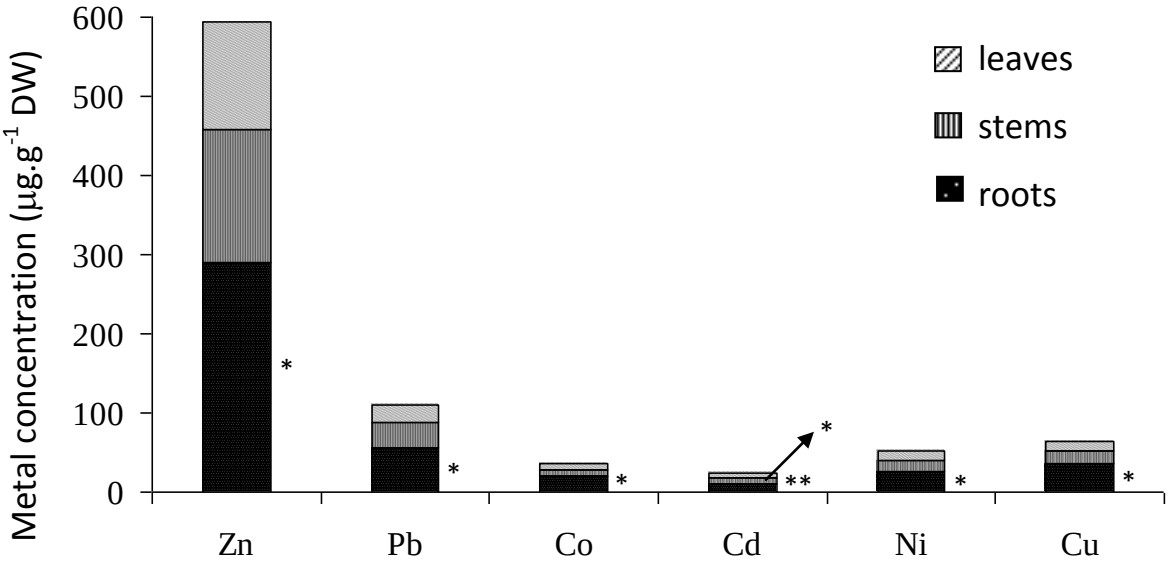


Figure 1. Metal concentrations (mg.g^{-1} DW) on *H. portulacoides* roots, stems and leaves, as a sum of all extracted fractions. (* and ** mean statistically different metal concentrations; Two way ANOVA, p>0.05 and Newman Keuls' test for Post-Hoc).

Table 2 – Translocation factor (TF) of each metal, calculated by the ratio of [metal] $_{leaves}$/[metal] $_{root}$

		Translocation factor			
Sampling	**Treatment**	**Zn**	**Cu**	**Ni**	**Cd**
T0	**1 - 5**	0.4	0.5	2.3	2.1
T1	**1**	0.1	0.1	1.3	0.6
	2	0.2	0.1	1.0	0.8
	3	0.1	0.2	1.3	3.9
	4	0.1	0.2	1.3	0.3
	5	0.2	0.2	1.7	0.6
T2	**1**	0.1	0.1	0.3	0.7
	2	0.2	0.3	0.5	0.3
	3	0.1	0.1	0.4	0.2
	4	0.2	0.4	0.6	0.8
	5	0.1	0.1	0.6	0.5

Regarding metal compartmentation in cell constituents, there was no statistical interaction between the plant organ and extracted fraction for each metal (Two way ANOVA, $p>0.05$). The metal concentrations at each extracted fraction for roots, stems and leaves of *H. portulacoides* is shown on Table 1. This plant presented significantly higher Zn concentration in the proteic fraction and the lower value was detected in cellulosis, with all other fractions presenting intermediate concentrations of Zn (Figure 2). The highest Pb percentage occurs in ethanolic and polissacaridic fractions. Co, Cd and Ni were mostly accumulated in the polissacaridic fraction in all plant organs, and cellullosis was the fraction with lower values of these metals. Cu was mostly accumulated in ethanolic, proteic and pectic fractions.

Considering the extracted fractions, and in order to simplify, metal location in the plant can be divided in three sections: cell wall, proteic fraction and intracellular location (the soluble metals). Cell wall includes the pectic, polissacaridic, lenhinic and cellulosic fractions. The proteins exact location in the cell cannot be determined by this extraction method. Intracellular metals include those extracted by ethanol and demineralised water (Figures 3 and 4). Roots accumulate on average 21% of metals intracellularly, 14% is retained in the proteic fraction and 65% is in the cell wall. In stems, on average 25% of metals are retained inside the cell, 20% the proteic fraction and the cell wall retains 55%. The highest percentage of metals accumulated intracellular occurs in leaves (32% average for all metals) and proteic fraction presents 15%. Leaves' cell walls retain 53% of metals. Co, Cd and Ni are the metals whose higher percentage is located in the cell wall of leaves stems and roots, whereas Pb presented the highest intracellular percentage. Zn was the metal presenting the highest percentage stored in the proteic fraction (Figure 3).

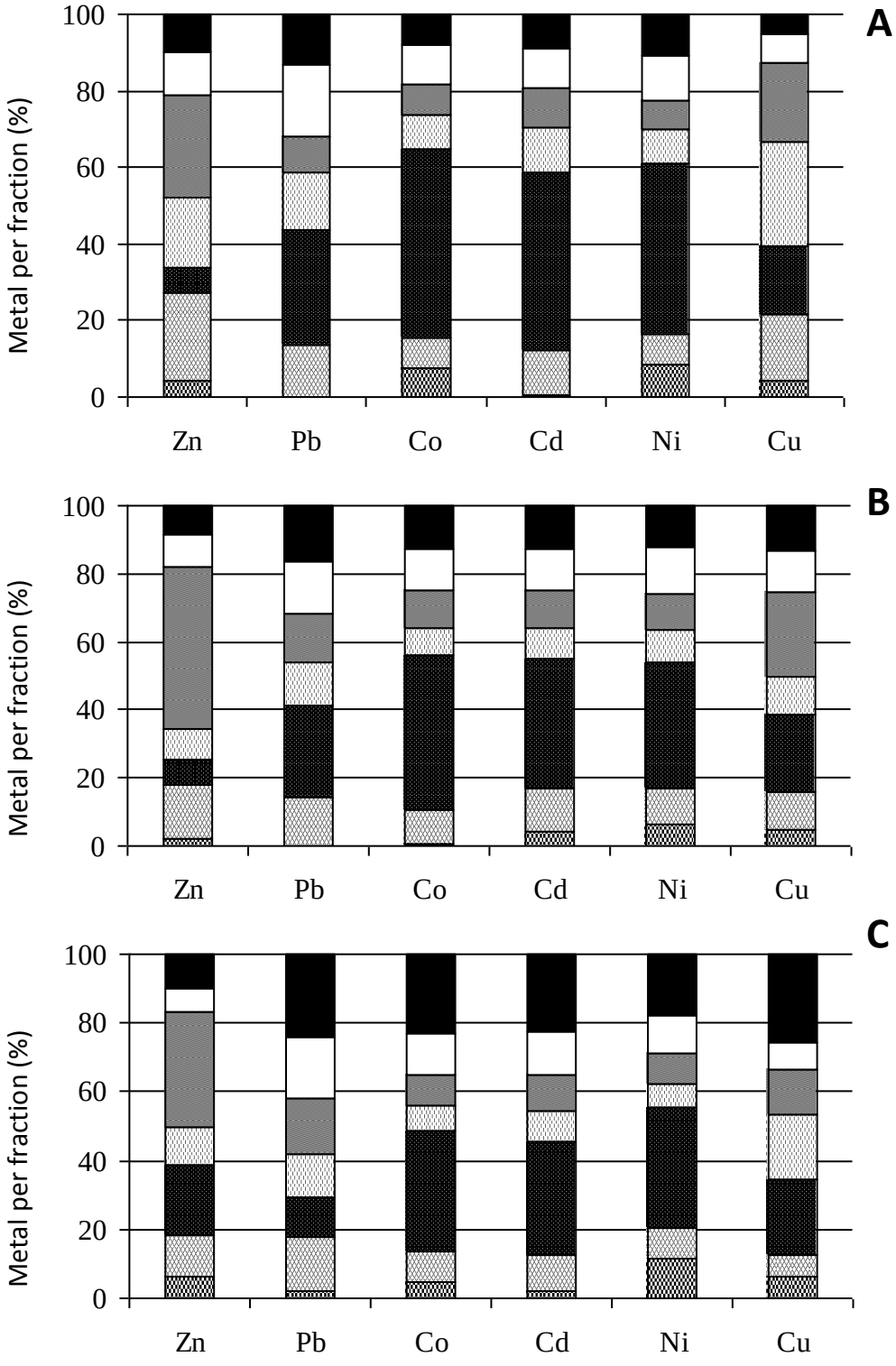


Figure 2. Metal concentrations (%) (average; n=3) on different fractions of *H. portulacoides* roots (A), stems (B), and leaves (C). The fractions, from top to down, are ethanolic (■), aqueous (□), proteic (▩), pectic (▥), polissacaridic (⁘), lenhinic (◇) and cellulosic (▪).

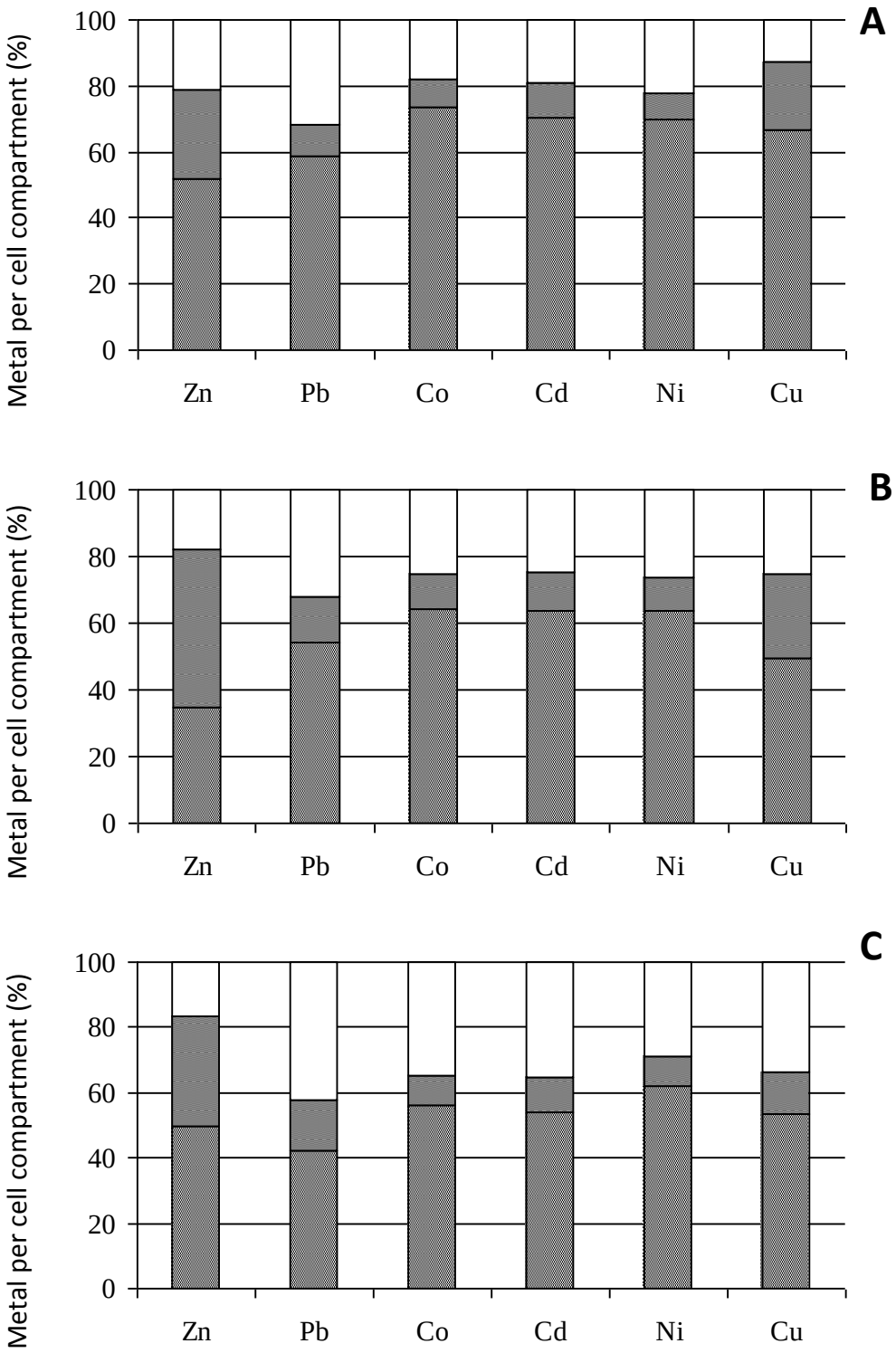


Figure 3. Metal organic ligands (%) (average; n=3) located intracellularly (☐) (ethanolic + aqueous fraction), on the proteic fraction (▨) and on the cell wall (▩) (pectic + polissacaridic + lenhinic +

cellulosic fractions) of *H. portulacoides* roots (A), stems (B) and leaves (C), corresponding to extra- and intra-cellular location.

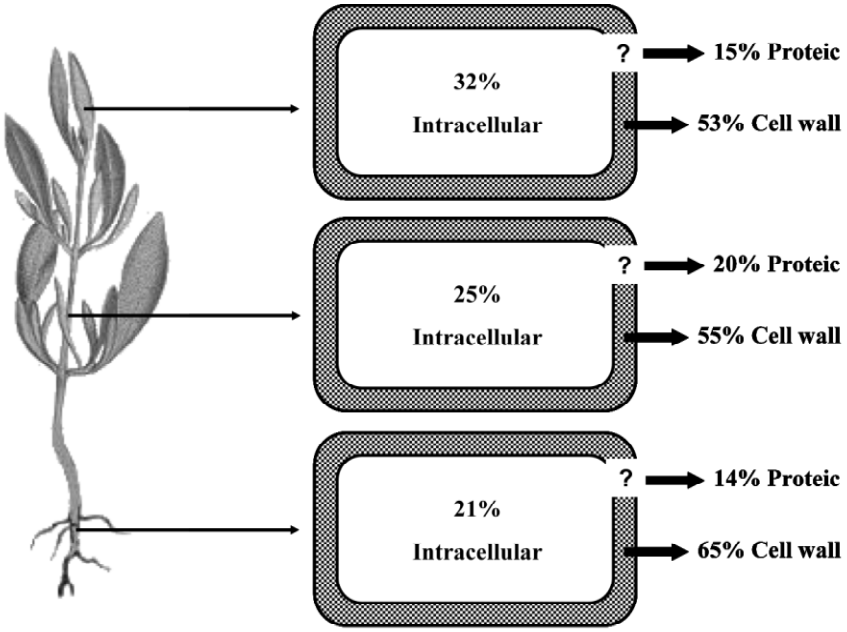


Figure 4. Metal distribution and compartmentation (cell wall ( ), proteic fraction, and intracellular location ( ) in *Halimione portulacoides* leaves, stems and roots, in the Tagus estuary (Portugal).

Discussion

The results show that roots of *H. portulacoides* accumulate much more metals than aboveground parts, which is in accordance with previous works from the Tagus estuary (Caçador et al., 2000; Reboreda et al., 2006). These results were also registered for Cd, Cu, Zn and Pb in several plant species (namely *Phragmites australis*), by Stoltz and Greger (2002) and in other aquatic macrophytes from Australia (Cardwell et al., 2002). In Ireland, *Spartina* spp. also present higher Cu and Pb concentrations in belowground material than in aboveground (Fitzgerald et al., 2003); and the same results were obtained for zinc in a wetland grass (*Glyceria fluitans*) of five european populations (Matthews et al., 2004). The limited mobility of the metals once inside the salt marsh plant (Deng et al., 2004) may be the explanation for the fact that metals are essentially accumulated in belowground rather than in the aboveground part of salt marsh plants. This is also traduced by translocation factors lower than 1, which was seen for almost all the studied metals, with exception of Zn and Cd. The high mobility and

bioavailability of Zn and Cd (Kiekens, 1995) may explain the high translocation factors observed for these two metals, both when considering total aboveground or leaves and stems separately, which results in higher metal concentrations in aboveground rather than in roots of *H. portulacoides*. According to McGrath (1995), once inside the plant, Ni is considered to have high mobility, which is in agreement with the TF value of 0.95 for aboveground material. The lowest TFs, registered for Cu which is a relative immobile metal in plants (Baker and Senft, 1995), traduce the higher accumulation of metals in the roots, instead of being translocated to the leaves of *H. portulacoides.*

Uptake and accumulation of metals by salt marsh plants depend on many factors such as plant species, age and growth stage of the plants, seasonal variations, existence of iron plaques on the roots, level of metal contamination in a specific local, soil properties, tidal inundations, salinity; and then metal characteristics influence the absorption, accumulation and translocation of metals (see Fitzgerald et al., 2003; Deng et al., 2004). Accordingly, plants have developed several strategies to survive in heavy metal contaminated soils. These strategies can be extracellular (mycorrhizas restrict metal movement to roots, metal binding to cell wall and extracellular exudates), at plasma membrane level (reducing uptake of heavy metals across plasma membrane, active efflux pumping of metals into the apoplast, that have entered the cytosol), and within the protoplast (chelation of metals by organic acids, amino acids or peptides; repair and protection of plasma membrane under stress conditions; transport and accumulation of metals in vacuole) (review Hall, 2002; Carvalho et al., 2006). Tolerance mechanisms for Zn and Ni have been explained by its complexation with organic acids in the cell vacuoles (Marschner, 1995). In the well known hyperaccumulator *Thlaspi caerulescens*, whereas roots accumulate Cd both in the apoplast, binding to cell wall components, and inside cells, proceeding in this way to detoxification, leaves use vacuoles as the main compartment for Cd storage and detoxification (Wójcik et al., 2005). According to Wójcik et al. (2005) the main mechanism for Cd detoxification in plants is the accumulation of phytochelatins.

The metal compartmentation in plants depends on several factors such as the metal, plant and nutrient availability (Barceló et al., 2004), and the aim of this study was to compare six metals compartmentation in only one plant species. As a whole, in *H. portulacoides* all studied metals presented higher percentage bind to cell wall compounds, both in roots, stems and leaves. However, the distribution of metals in different cell wall extracted fractions varied with metal, as mentioned in the results section, which may be related with own properties of the metal and characteristics of the studied plant.

This work shows that metal content in the intracellular compartment of *H. portulacoides* is much lower than the total of metal retained by the plant. Considering these data and the existence of many detoxifying mechanisms known for other plant species for preventing the plants from toxicity, it can be stated that this compartmentation and detoxifying mechanisms are crucial for these plants metal accumulation capacity and tolerance. This may show that the high heavy metal levels in the salt marsh sediment do not causes toxicity to the plants because plant immobilizes them outside key metabolic sites. Thus, they are not only accumulated intracellularly in the plant, but in different cell compartments and bind to different cell compounds (cell wall + proteic fraction + intracellular). This compartmentation may contribute and may be crucial to the surviving of salt marsh plants in highly metal contaminated salt marshes. Altogether, this study contributes to a better knowledge of different compartmentation of Zn, Pb, Cu, Ni, Co and Cd inside *H. portulacoides* cells and also in different plant organs (leaves, stems and roots). Afterwards, once plant dies leaching process turns metals binded to intracellular and proteic fractions (nitrogen compounds) earlier available, corresponding almost to 50% of metals within plants. In turn, cell wall structures, composed by lenhinic compounds (carbon) decompose slowly and metals (the other 50%) will be available later. Thus, compartmentation of metals within plants also influences and conditionates the bioavailability of metals in salt marshes.

References

Baker, D.E., Senft, J.P., 1995. Copper. In: Alloway, B.J. (Ed.), Heavy metals in soils. 2nd Edition, Blackie Academic & Professional, London, UK, pp. 179-205.

Barceló, J., Poschenrieder, Ch., 2004. Structural and Ultrastructural Changes in Heavy Metal Exposed Plants. In Prasad, M.N.V. (Ed.), Heavy Metal Stress in Plants – From Biomolecules to Ecosystems, 2nd edition, Springer, Heidelberg, pp. 223-248.

Caçador, I., Vale, C., Catarino, F., 2000. Seasonal variation of Zn, Pb, Cu and Cd concentrations in the root±sediment system of *Spartina maritima* and *Halimione portulacoides* from Tagus estuary salt marshes. Marine Environmental Research 49, 279-290.

Cardwell, A.J., Hawker, D.W., Greenway, M., 2002. Metal accumulation in aquatic macrophytes from southeast Queensland, Austrália. Chemosphere 48, 653–663.

Carrier, P., Baryla, A., Havaux M., 2003. Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contaminated soil. Planta 216, 939–950.

Carvalho, L.M., Caçador, I., Martins-Loução, M.A., 2006. Arbuscular mycorrhizal fungi enhance root cadmium and copper accumulation in the roots of the salt marsh plant *Aster tripolium* L. Plant and Soil 285, 161-169.

Deng, H., Yea, Z.H., Wong, M.H., 2004. Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-contaminated sites in China. Environmental Pollution 132, 29-40.

Farago, M.E., Pitt, M.J., 1977. Plants which accumulate metals. Part II. An investigation of the soluble zinc containing extracts from two Australian species. Inorganica Chimica Acta 24, 127-130.

Fitzgerald, E.J., Caffrey, J.M., Nesaratnam, S.T., McLoughlin, P., 2003. Copper and lead concentrations in salt marsh plants on the suir Estuary, Ireland. Environmental Pollution 123, 67-74.

Greger, M., 1999. Metal Availability and Bioconcentration in Plants. In: Prasad, M.N.V., Hagemeyer, J. (Eds.), Heavy Metal Stress in Plants: From Molecule to Ecosystems. Springer-Verlag, Berlin, Heidelberg, Germany.

Greger, M., 2004. Metal availability, Uptake, Transport and Accumulation in Plants. In Prasad, M.N.V. (Ed.), Heavy Metal Stress in Plants – From Biomolecules to Ecosystems, 2nd edition, Springer-Verlag, Berlin, Heidelberg, Germany, pp. 1-27.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. Journal of Experimental Botany 53(366), 1-11.

Kiekens, L., 1995. Zinc. In: Alloway, B.J. (Ed.) Heavy metals in soils. 2nd Edition, Blackie Academic & Professional, London, UK, pp. 284-305.

Lozano-Rodriguez, E., Hernandéz, L.E., Bonay, P., Carpena-Ruiz, R.O., 1997. Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. Journal of Experimental Botany 48, 123-128.

Marschner, H., 1995. Mineral Nutrition of Higher Plants. 2nd Edition, Academic Press Limited, London, UK.

Matthews, D.J., Moran, B.M., McCabe, P.F., Otte, M.L., 2004. Zinc tolerance, uptake, accumulation and distribution in plants and protoplasts of five European populations of the wetland grass *Glyceria fluitans.* Aquatic Botany 80, 39-52.

McCabe, O.M., Baldwin, J.L., Otte, M.L., 2001. Metal tolerance in wetland plants? Minerva Biotecnologica 13, 141–149.

McGrath, S.P., 1995. Chromium and Nickel. In: Alloway, B.J. (Ed.) Heavy metals in soils. 2nd Edition, Blackie Academic & Professional, London, UK, 152-178.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh capacity retention for heavy metals. Environmental Pollution 146, 147-154.

Salt, D.E., Prince, R.C., Pickering, I.J., 2002. Chemical speciation of accumulated metals in plants: evidence from X-ray absorption spectroscopy. Microchemical Journal 71, 255-259.

Stoltz, E., Greger, M., 2002. Accumulation properties of As, Cd, Cu, Pb and Zn by four wetland plant species growing on submerged mine tailings. Environmental and Experimental Botany 47, 271-280.

Wójcik, M., Vangronsveld, J., D'Haen, J., Tukiendorf, A., 2005. Cadmium tolerance in *Thlaspi caerulescens.* II. Localization of cadmium in *Thlaspi caerulescens.* Environmental and Experimental Botany 53, 163-171.

CHAPTER III

Multiple Stressors: N and Metals

CHAPTER III - MULTIPLE STRESSORS: N AND METALS

Introduction – Multiple stressors

Due to increasing global population, salt marshes have been subjected to multiple stressors such as increasing nutrient loadings and historical contamination.

Toxic compounds accumulate through complex physical and chemical adsorption mechanisms depending on the properties of the adsorbed compounds and the nature of the sediment. Although differentiation between metals depends upon the chemical properties of the metals and their compounds and upon the biological properties of the organisms at risk, metals can be grouped according to the Lewis acid classification (Duffus, 2002). Class A (hard) metals: Li, Be, Na, Mg, Al, K, Ca, Sc, Ti, Fe(III), Rb, Sr ,Y, Zr, Cs, Ba, La, Hf, Fr, Ra, Ac, Th; Class B (soft) metals: Cu(I), Pd, Ag, Cd, Ir, Pt, Au, Hg, Ti, Pb(II); Borderline (intermediate) metals: V, Cr, Mn, Fe(II), Co, Ni, Cu(II), Zn, Rh, Pb(IV), Sn. Arsenic (As) is a semimetal, i.e., an element that has the physical appearance and properties of a metal but behaves chemically like a nonmetal (Duffus, 2002).

Since the mid of last century, the anthropogenic sources of metals into the aquatic systems have been reduced due to legal restrictive rules. However, metals contaminated sediments from the past, i.e, "*historical contamination*" are still cause for concern due to their potential release into other environmental matrices. For instance, the replacement of the native *Scirpus maritimus* and *Phragmites australis* by *S. alterniflora* in a salt marsh at Yangtze River estuary (China) would significantly improve the magnitude of nutrient cycling and bioavailability of trace metals in the salt marsh, and eventually deliver more toxic metals into the water column in the estuary (Quan et al., 2007). On the other hand, salt marsh sediments are known to retain different kind of contaminants, namely heavy metals, chlorinated hydrocarbons, petroleum hydrocarbons (e.g. Reddy et al., 2002, Valiela et al., 2004), as well as nutrients (Valiela and Cole, 2002; Valiela et al., 2004). As previously mentioned, a *stressor* can be defined as "*a factor that extends homeostatic or protective processes beyond thelimits of the normal physiological or ecological range leading to reduced fitness*" (Sibly and Calow, 1989, and Moore et al., 2002 in Segner, 2007). Thus, the challenge now is to understand how the effect of a stressor is determined by the interacting effect with another stressor and how the risk of an interaction between chemical, physical and biological stressors can be assessed and predicted.

References

Segner, E., 2007. Ecotoxicology – How to assess the impact of toxicants in a Multi-Factorial Environment? In: Mothersill, C.; Mosse, I.; Seymour, C. (Eds.) Multiple Stressors: A Challenge for the Future 484pp.

Valiela, I., Cole, M.L., 2002. Comparative evidence that salt marshes and mangroves may protect seagrass meadows from land-derived nitrogen loads. Ecosystems 5, 92–102.

Duffus, J., 2002. "Heavy Metals" – A Meaningless Term? (IUPAC Technical Report), Pure and Applied Chemistry 74(5), 793–807.

Quan W.M., Han, J.D., Shen, A.L., Ping, X.Y., Qian, P.L., Li, C.J., Shi, L.Y., Chen, Y.Q., 2007. Uptake and distribution of N, P and heavy metals in three dominant salt marsh macrophytes from Yangtze River estuary, China. Marine Environmental Research 64, 21-37.

Reddy, C., Englinton, T.I., Hounshell, A., White, H.K., Xu, L., Gaines, R.B., Frysinger, G.S., 2002. The WestFalmouth oil spill after thirty years: the persistence of petroleum hydrocarbons in marsh sediments. Environmental Science Technology. 36, 4754– 4760.

Valiela, I., Rutecki, D., Fox, S., 2004. Salt marshes: biological controls of food webs in a diminishing environment. Journal of Experimental Marine Biology and Ecology 300, 131– 159.

Case studies

1. Influence of multiple stressors on the auto-remediation processes occurring in salt marshes

Abstract

Due to increasing global population, salt marshes have been subjected to multiple stressors such as increasing nutrient loadings and historical metal contamination. In order to better understand how does the salt marsh plants auto-remediation capacity (phytoaccumulation of metals) is affected by cultural eutrophication, an experiment was performed under controlled conditions. Plants were exposure to equal metal concentrations (Zn, Cu, and Ni – micronutrients, and Cd – class B metal) simulating historical contamination and three different concentrations of nitrogen (nitrate) simulating steps of cultural eutrophication. According to our study, under the tested concentrations, cultural eutrophication does not seem to affect Zn, Cu and Ni phytoremediation of *H. portulacoides,* but the ecosystem service of Cd phytoremediation seems to be promoted. Nevertheless, Cd high toxicity and bioaccumulation should be taken into account, as well as the vulnerability of salt marsh ecosystems, whose reduction will have drastic consequences to the ecosystem health.

Keywords: salt marshes, eutrophication, metals, multiple stressors, phytoaccumulation, *Halimione portulacoides*

Introduction

Salt marshes are valuable ecosystems, providing many benefits to human population as well as habitats to other estuarine biological communities. These ecosystems are very productive and dynamic and have crucial functions such as protection of coastal areas from erosion and floods, increase water quality by enhancing sediment deposition and by promoting pollutants (e.g. metals) sequestration (Constanza et al., 1997; McLusky and Elliott, 2004). Parallel to the industry development and worldwide increase of human population in coastal areas during the last century, salt marshes have been exposed to industrial and other anthropogenic pressures, especially when cities developed within the estuarine areas. Subsequently, estuaries and salt marshes in particular have been subdued to metals contamination (e.g. Vega et al., 2009). Salt marsh plants are well known by their capacity to accumulate and retain metals (e.g. Ghosh and Singh, 2005; Sousa et al., 2008a). Moreover,

cultural eutrophication has been a common process within coastal systems all over the world, (e.g. Kemp et al., 2005; Lillebø et al., 2007). As an auto-remediation process, salt marsh halophytes may contribute, as well, to the reduction of eutrophication through nitrogen uptake for biomass production (Sousa et al., 2008b; Sousa et al., 2010) and enhancing denitrification (Valiela et al., 2000; Sousa et al., unpublished data). However, whether high loads of nitrogen, i.e. cultural eutrophication, in sediments historically contaminated can affect the retention capacity of metals by salt marsh halophytes is scarcely known (e.g. Carvalho et al., 1998; Bose et al., 2008).

The main objective of this work is to analyse how cultural eutrophication may affect the auto-remediation process in historical metal contaminated salt marshes (effects of multiple stressors). To address this question, *Halimione portulacoides* (L.) Aellen was the chosen halophyte since it colonizes low and mid-marsh areas of European salt marshes, being one of the most abundant species (Bouchard et al., 1998). The chosen metals, zinc (Zn), copper (Cu) and nickel (Ni) are essential micro-nutrients but can produce toxic effects above tolerable concentrations by plants, while cadmium (Cd) can induce toxicity in plants even in low concentrations (e.g. Duarte et al., 2010). In this approach two hypothesis will be tested: 1) does the *H. portulacoides* ability to sequestrate through phytoaccumulation of Zn, Cu, Ni and Cd depend on the supply of nitrogen?; 2) is the auto-remediation process of *H. portulacoides,* in sediments historically contaminated with metals, affected by the availability of nitrogen?

Materials and methods

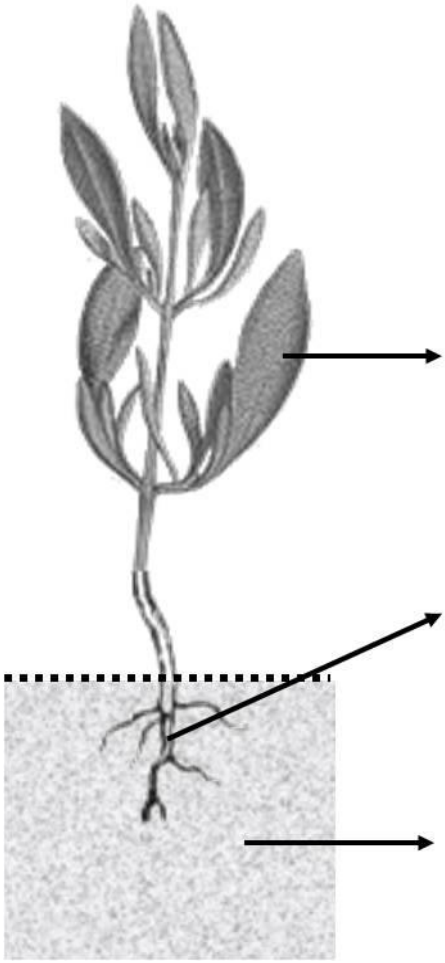
These hypotheses were tested through a glasshouse experiment as described in Table 1. *H. portulacoides* specimens were collected in a Tagus estuary salt marsh (38^0 40’ 10.32’’ N, 9^0 00’ 13.20’’ W), in order to make grafts. These grafts were kept in Hoagland’s nutrient solution, and placed in a glasshouse for about two months, allowing to grow new root biomass. Afterwards, plants were transplanted to individual pots filled up with estuarine sediment characterized by low nitrogen and metals concentrations. The initial concentrations of metal in sediment and in plant organs are shown in Figure 1. In the experimental set-up five conditions were tested, in which metal concentrations used intended to simulate a historically contaminated salt marsh (Caçador et al., 1996; Reboreda and Caçador, 2007) and the chosen nitrogen levels reproduced cultural eutrophication in estuarine systems (e.g. Lillebø et al., 2007) (Table 1).

Table 1. Experimental design and characterization of the rhizosediment and plant in the beginning of the experiment (T0). Metal concentrations are indicated in $\mu g.g^{-1}DW$; three replicates (n = 3) were analyzed per *treatment* X *sampling date* combination.

Experimental design		
Treatment	**1**	PES[a] (Control)
	2	PES + Metals[b]
	3	PES + Metals[b] + 10 $\mu mol.l^{-1}$ NO_3-N
	4	PES + Metals[b] + 50 $\mu mol.l^{-1}$ NO_3-N
	5	PES + Metals[b] + 100 $\mu mol.l^{-1}$ NO_3-N
Sampling	**T1**	8 weeks (2 months)
	T2	25 weeks (≈ 6 months)

[a] Provasoli enriched seawater (modification of L. Provasoli (1963), in Bold and Wynne, 1978)

[b] Metals concentration: 410 µg $Zn.g^{-1}DW$ + 70 µg $Cu.g^{-1}DW$ + 4 µg $Cd.g^{-1}DW$ + 30 µg $Ni.g^{-1}DW$



Metal concentrations ($\mu g.g^{-1}DW$)	
Zn	16.8±4.3
Cu	21.8±6.8
Ni	2.9±0.6
Cd	1.4±0.2
Zn	39.2±12.1
Cu	42.2±9.9
Ni	1.3±0.3
Cd	0.6±0.3
Zn	83.8±11.8
Cu	11.4±1.5
Ni	9.2±2.1
Cd	0.8±0.1

Figure 1. Metal concentrations ($\mu g.g^{-1}DW$, avrge ± SD, n=3) in *Halimione portulacoides'* leaves and roots, and in the rhizosediment.

Sampling and laboratory procedure

After two (T1) and six (T2) months, three plant replicates (3 pots each with an individual plant) were rinsed with distilled water, roots and leaves were separated and dried at 60 °C for 72 h and then ground with liquid nitrogen in a homogenizer. Dry homogenized plant material (about 100 mg aliquots) was subdue to an acid digestion by adding 2 ml HNO3/HClO4 (7:1, v/v) in a acid washed teflon reactor placed in a furnace for 3h at 110 °C. After cooling, the solution was filtered through Whatman 42 (pore Ø 2.5 μm) filters and diluted with distilled water up to 5 ml. Total concentrations of metals in sediments were determined using 100 mg of homogenized-dried aliquots following the previous procedure except for the acid solution, 2 ml HNO3/HCl (3:1, v/v). Concentrations of Zn, Cu, Ni and Cd in the extracts were determined by air-acetylene flame atomic absorption spectroscopy (VARIAN Spectr AA-50) and a manual microinjection method. Detection limits were 0.01, 0.10, 0.05 and 0.05 $\mu g\ g^{-1}$, for Zn, Cu, Ni and Cd respectively, for all the analytical procedures used. Analytical quality control was performed by using certified reference material (sewage sludge - CRM 145 and CRM 146 - and plant material - BCR 62 – *Olea europaea*) in parallel with samples. For all metals investigated, obtained values were consistently within the ranges of certified values ($p < 0.05$). The detection limits of the AAS analysis of plants were in $mg\ kg^{-1}$ dry weight for Zn (0.33), Cd (0.03), Ni (0.15) and Cu (0.03).

Statistical analyses

One-way ANOVA (analysis of variance) was performed to test for differences in plants' metal content between leaves and roots (two levels) at T0. Two-way ANOVA was performed for each metal to test for differences in plants' metal concentration between sampling dates (two levels, T1 and T2) and applied treatments (five levels, treatment 1 to 5). ANOVA assumptions were assured: normality of the data (Kolmogorov-Smirnov test) and homogeneity of variances (Cochran's Q test; data were transformed when necessary). Post-hoc comparisons were performed by Newman-Keuls test at $\alpha=0.05$ significance level. Analyses were performed with STATISTICA 9.0 software package. The translocation factor (TF) expressing the metals translocation within the plant (from roots to the leaves) was calculated by the ratio of $[metal]_{leaves}/[metal]_{roots}$ (Deng et al., 2004).

Results

Regarding initial concentrations of metals, both Zn and Ni (concentrations) were higher in sediment than in the plant organs (roots and leaves). Copper (Cu) concentrations were higher in roots, followed by the concentrations in leaves. Regarding Cd, concentrations were comparatively higher in leaves (Figure 1). Metal concentrations in *H. portulacoides* organs (roots and leaves) throughout the experimental period are shown in Figure 2. In roots, Zn concentration did not show differences between sampling dates, neither between treatments ($p > 0.05$). Regarding leaves, Zn concentrations did not show differences among sampling dates ($p > 0.05$), but there were significant differences among treatments 1 (control) and 4 (metal + 50 µM NO_3-N), ($F = 2.89$, $p < 0.05$). The increased nitrogen availability does not seem to influence the uptake of Zn from the sediment nor the translocation of Zn from roots to leaves (Table 2). Cu concentration in roots showed significant differences between sampling dates ($F = 163.16$, $p < 0.0001$), whereas in leaves there was interaction between treatments and sampling dates ($F = 6.19$, $p < 0.01$), i.e., Cu concentration in leaves regarding treatments 2 (metals) and 4 (metal + 50 µM NO_3-N) from T2 (time 2 = 6 months) were equal, but different from all the other treatments (control, metal + 10 µM NO_3-N, metal + 100 µM NO_3-N). The increased nitrogen availability does not seem to influence the uptake of Cu from the sediment nor the translocation of Cu from roots to leaves (Table 2). Ni concentrations in roots were significantly different between sampling dates ($F = 17.74$, $p < 0.001$) and in leaves significant differences occurred between treatment 1 (control) and 5 (metal + 100 µM NO_3-N) ($F = 3.34$, $p < 0.05$). Ni translocation from roots to the leaves seems to be affected by the high availability of nitrogen (Table 2). Cd concentrations in roots showed interaction between sampling dates and treatments ($F = 7.04$ $p < 0.01$): all treatments from T1 (time 1 = 2 months) and T2 (time 2 = 6 months) were significantly higher than treatment 3 (metal + 10 µM NO_3-N) from T1 (time 1), except treatment 1 (control) for T2 (time 2). Interestingly, treatments with nitrogen enrichment seem to affect the uptake of Cd by roots, after 6 months of exposure. Regarding leaves, treatments 4 (metal + 50 µM NO_3-N) and 5 (metal + 100 µM NO_3-N) from T2 (time 2 = 6 months) were significantly different from all the other ($F = 7.65$, $p < 0.001$), meaning that Cd translocation from roots to the leaves also seems to be affected by the increase of available nitrogen (Table 2).

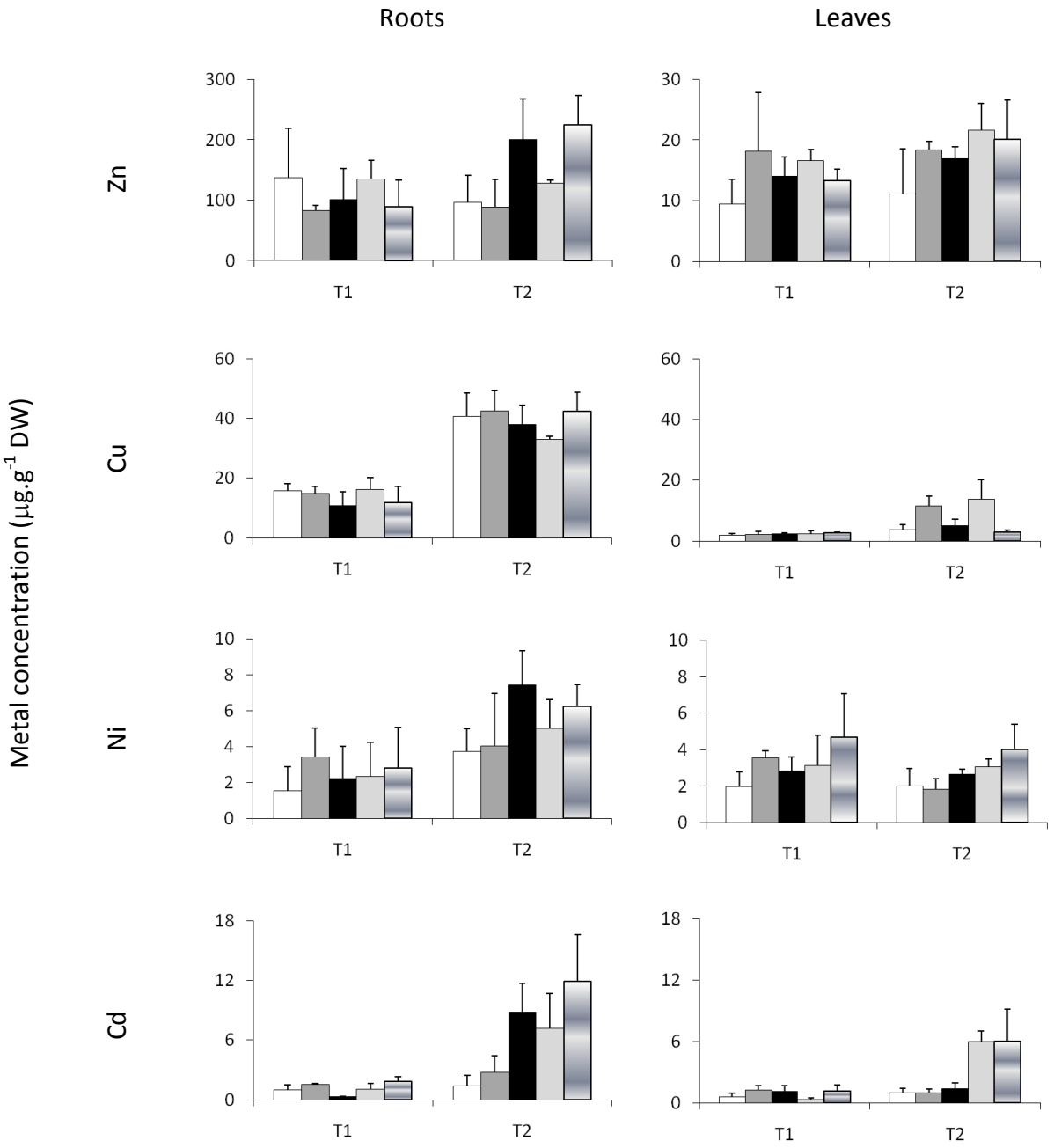


Figure 2. Metal concentrations (Zn, Cu, Ni, Cd) in *H. portulacoides*' roots and leaves (avrge ± SD, n=3). Sampling dates are represented as T1 and T2; each coloured column is a different applied treatment (1 - PES (Control), 2 - PES + Metals[a], 3 - PES + Metals[a] + 10 µmol.l^{-1} NO_3-N, 4 - PES + Metals[a] + 50 µmol.l^{-1} NO_3-N, 5 - PES + Metals[a] + 100 µmol.l^{-1} NO_3-N; [a] Metals' concentration: 410 µg Zn.g^{-1}DW + 70 µg Cu.g^{-1}DW + 4 µg Cd.g^{-1}DW + 30 µg Ni.g^{-1}DW).

Table 2 – Translocation factor (TF) of each metal, calculated by the ratio of [metal] $_{leaves}$/[metal] $_{root}$

		Translocation factor			
Sampling	**Treatment**	**Zn**	**Cu**	**Ni**	**Cd**
T0	**1 - 5**	0.4	0.5	2.3	2.1
T1	**1**	0.1	0.1	1.3	0.6
	2	0.2	0.1	1.0	0.8
	3	0.1	0.2	1.3	3.9
	4	0.1	0.2	1.3	0.3
	5	0.2	0.2	1.7	0.6
T2	**1**	0.1	0.1	0.3	0.7
	2	0.2	0.3	0.5	0.3
	3	0.1	0.1	0.4	0.2
	4	0.2	0.4	0.6	0.8
	5	0.1	0.1	0.6	0.5

Discussion

Regarding plant organs, *H. portulacoides* uptakes and sequestrates more Zn, Cu and Cd in roots rather than in leaves, as also reported by other authors for this species and for other rooted macrophytes (e.g. Bose et al., 2008; Bonanno and Giudice, 2010; Duarte et al., 2010). Accordingly, translocation factor from roots to leaves was generally lower than 1, except for Ni after 2 months. Thus, *H. portulacoides* roots may act as a barrier for translocation of Zn, Cu, Cd from roots to leaves, preventing toxicity – metal compartmentalization – as reported in other works (Bose et al., 2008; Caçador et al., 2009). In addition, several factors such as plant species, metal characteristics and contamination of the site, tidal inundations, salinity, organic matter, etc.; determine the uptake, accumulation and translocation of metals by salt marsh plants (e.g. Deng et al., 2004; Bose et al., 2008). Moreover, constraint in metal uptake and accumulation in aboveground organs may be due to exclusion mechanisms developed in plant species from contaminated soils, as discussed by Stoltz and Greger (2002). The reduced mobility of the metals inside the plant may be another reason for this higher metal accumulation in the roots (Deng et al., 2004; Sousa et al., 2008a; Bonanno and Giudice, 2010).

The uptake of the borderline (intermediate electron acceptors) micronutrients (essential for plants in metabolic and physiological reactions, present in low concentrations) (Rengel, 2004) - Zn, Cu and Ni - by *H. portulacoides*' roots (phytoaccumulation) does not seem to depend on the nitrogen availability neither, for Zn, on the time of exposure (at least for 6 months). Cu and Ni uptake by *H. portulacoides* increases after 6 months of exposure, being phytoaccumulated in the roots. Regarding the translocation of Zn, Cu and Ni to the aboveground organs (leaves), it does not seem to increase with time of exposure neither with the availability of nitrogen (at least under the tested concentrations). Concerning Ni accumulation in leaves, it is higher when both metals and high nitrogen availability are present. Moreover, lower nitrogen availability may not influence Ni accumulation in leaves. The absence of correlation between Mn, Cu, Zn, Cr, Ni, Pb phytoaccumulation and nitrogen availability was recorded for other rooted macrophytes (Bose et al., 2008) and between Zn, Cu, Cd and nitrogen in the sediment for *Juncus effusus* and *Equisetum ramosisti* (Deng et al., 2004). Thus, as stated for other species, nitrogen availability in the sediment may not be the main factor determining the Zn, Cu and Ni uptake by *H. portulacoides*.

Cd is a class B (soft acceptor; has preference for binding with ligands containing sulfur and nitrogen) and highly toxic element without any known physiological function in plant acceptors. Regarding the accumulation of Cd in roots and translocation to leaves, the increase of nitrogen availability enhanced phytoaccumulation. Some works have shown that environmental conditions and the availability of some metals determine the uptake of other metals. Namely, *Typha angustata* decreased the Cu uptake in the presence of higher Zn concentrations than Cu (Bose et al., 2008) and *Medicago sativa* reduced Cu uptake in the presence of Zn (Peralta-Videa et al., 2003), having an antagonistic effect on its uptake by plants. Accordingly, it seems there was a synergistic effect (between metals contamination - Zn, Cu, Ni, Cd - and nitrogen availability) on the Cd uptake by *H. portulacoides'* roots and its translocation to the leaves. Thus, in the presence of the mentioned multiple stressors (metals and nitrogen), *H. portulacoides* seems to increase its phytoaccumulation capacity for Cd. The different behaviour among studied metals may be due to different characteristics of each metal and their role in the plants. Zn is essential element to all plants, forms organic complexes such as amino and organic acids and is required for the activity of various types of enzymes; Cu is essential to plant growth and its biochemical function is mainly to be a cofactor in enzymes; Ni is component of enzyme urease and essential for its functioning. Cd is doesn't have a know function for plants.

To sum up, increasing nitrogen concentration in the presence of high metal concentrations does not affect the uptake of Zn, Cu and Ni by *H. portulacoides* roots and

translocation to the leaves. On the other hand, uptake of Cd by *H. portulacoides* roots is positively affected by the increase availability of nitrogen, meaning that cultural eutrophication may enhance this ecosystem service (Cd phytoaccumulation, at least within the tested concentrations). According to our study, there is no effect of these multiple stressors (cultural eutrophication and historical contamination with Zn, Cu, Ni and Cd) on this ecosystem service provided by *H. portulacoides* (Zn, Cu and Ni phytoremediation). Cd is a priority substance under the Water Framework Directive legislation (Annex II, Directive 2008/105/EC) (http://ec.europa.eu/environment/water/water-dangersub), it is very toxic and is bioaccumulated throughout the food web. It is transferred from the water/sediments to the plants and also to mussels, oysters and fishes, constituting a threat to environmental, ecosystem and human health. Even though Cd phytoaccumulation does not seem to be affected in the presence of cultural eutrophication, it shouldn't be neglected Cd direct and indirect effects as a pollutant and the vulnerability of salt marsh ecosystems (e.g. Best et al., 2007), whose reduction will have drastic consequences to the ecosystem health.

References

Best, M., Massey, A., Prior, A., 2007. Developing a saltmarsh classification tool for the European water framework directive. Marine Pollution Bulletin 55, 205–214.

Bold, H.C., Wynne, M.J., 1978. Introduction to the algae: structure and reproduction. New Jersey, Prentice Hall, Inc. Englewood Cliffs, New Jersey.

Bonanno, G., Giudice, R.L., 2010. Heavy metal bioaccumulation by the organs of *Phragmites australis* (common reed) and their potential use as contamination indicators. Ecological Indicators 10, 639-645.

Bose, S.,Vedamati, J., Rai, V., Ramanathan, A.L., 2008. Metal uptake and transport by *Typha angustata* L. grown on metal contaminated waste amended soil: An implication of phytoremediation. Geoderma 145, 136-142.

Bouchard, V., Creach, V., Lefeuvre, J.C., Bertru, G., Mariotti, A., 1998. Fate of plant detritus in a European salt marsh dominated by Atriplex portulacoides (L.)Aellen. Hydrobiologia 373/374, 75-87.

Caçador, I., Caetano, M., Duarte, B., Vale, C., 2009. Stock and losses of trace metals from salt marsh plants. Marine Environmental Research 67, 75-82.

Caçador, I., Vale, C., Catarino, F.M., 1996. Accumulation of Zn, Pb, Cu, Cr and Ni in Sediments Between Roots of the Tagus Estuary Salt Marshes, Portugal. Estuarine, Coastal and Shelf Science 42, 393-403.

Carvalho, L.M., Caçador, I., Cruz, C., Martins-Loução, M.A., 1998. Acumulação de cobre em *Halimione portulacoides* (L.) Allen. Revista de Biologia 16(1-4): 185-193.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O' Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 353-360.

Deng, H., Yea, Z.H., Wong, M.H., 2004. Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-contaminated sites in China. Environmental Pollution 132, 29-40.

Duarte, B., Caetano, M., Vale, C., Caçador, I., 2010. Accumulation and biological cycling of heavy metal in four salt marsh species, from Tagus estuary (Portugal). Environmental Pollution 158, 1661-1668.

Ghosh, M., Singh, S.P., 2005. A review on phytoremediation of heavy metals and utilization of its byproducts. Applied Ecology and Environmental Research 3(1), 1-18.

Kemp, W.M., Boynton, W.R., Adolf, J.E., Boesch, D.F., Boicourt, W.C., Brush, G., Cornwell, J.C., Fisher, T.R., Glibert, P.M., Hagy, J.D., Harding, L.W., Houde, E. D., Kimmel, D.G., Miller, W.D., Newell, R.I.E., Roman, M.R., Smith, E.M., Stevenson, J.C., 2005. Eutrophication of Chesapeake Bay: historical trends and ecological interactions. Marine Ecology Progress Series 303, 1–29.

Lillebø, A.I., Teixeira, H., Pardal, M.A., Marques, J.C., 2007. Applying quality status criteria to a temperate estuary before and after the mitigation measures to reduce eutrophication symptoms. Estuarine, Coastal and Shelf Sciences 72, 177-187.

McLusky, D.S., Elliot, M., 2004. The Estuarine Ecosystem – Ecology, Threats, and Management, third ed. Oxford University Press.

Peralta-Videa, J.R., Gardea-Torresdey, J.L., Walton, J., Mackay, W.P., Duarte-Gardea, M., 2003. Effects of Zinc upon Tolerance and Heavy Metal Uptake in Alfalfa Plants (*Medicago sativa*). Bulletin of Environmental Contamination and Toxicology 70, 1036–1044.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh capacity retention for heavy metals. Environmental Pollution 146, 147-154.

Rengel, Z., 2004. Heavy metals as essential nutrients. In Prasad, M.N.V. (Ed.), Heavy Metal Stress in Plants – From Biomolecules to Ecosystems, 2nd edition, Springer-Verlag, Berlin, Heidelberg, Germany, pp. 271-294.

Sousa, A.I., Caçador, I., Lillebø, A.I., Pardal, M.A., 2008a. Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites. Chemosphere 70, 850–857.

Sousa, A.I., Lillebø, A.I, Caçador, I., Pardal, M.A., 2008b. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628–635.

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I., 2010. Productivity and nutrient cycling in salt marshes: Contribution to ecosystem health. Estuarine, Coastal and Shelf Science 87, 640-646.

Stoltz, E., Greger, M., 2002. Accumulation properties of As, Cd, Cu, Pb and Zn by four wetland plant species growing on submerged mine tailings. Environmental and Experimental Botany 47, 271-280.

Valiela, I., Cole, M.L., Mcclelland, J., Hauxwell, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes. In: Weinstein, M.P., Kreeger, D.A. (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, pp. 23–38.

Vega, F.A., Covelo, E.F., Reigosa, M.J., Andrade, M.L., 2009. Degradation of fuel oil in salt marsh soils affected by the Prestige oil spill. Journal of Hazardous Materials 166, 1020–1029.

2. Denitrification in salt marshes with different historical metal contamination: comparison of two temperate estuaries

Abstract

Since the mid of last century, the anthropogenic sources of metals into the aquatic systems have been reduced due to legal restrictive rules. However, metals contaminated sediments from the past, i.e, "*historical contamination*" are still cause for concern due to their potential release into other environmental matrices. In the present study we hypothesize that denitrification, as a service provided by *Spartina maritima* marshes may be affected by the presence of metals, namely Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and the metalloid As. Denitrification was quantified in two monospecific *S. maritima* marshes differently stressed by metals, using the ^{15}N-isotope pairing technique. Results showed that in winter in the compared marshes, denitrification was significantly lower in the non-contaminated marsh and always lower under light conditions. Under the studied conditions, daily denitrification was about 2285 ± 420 µmol N m^{-2} day^{-1} at the non-metal-contaminated *S. maritima* marsh and 11046 ± 7398 µmol N m^{-2} day^{-1} at the contaminated one. However, at the contaminated marsh the variability is much higher. Finally, this study contributes to evaluate the auto-remediation capacity of salt marshes through denitrification, regarding multiple stressors, *e.g.* "*cultural eutrophication*" and "*historical contamination*" with metals, but more comparable results will be valuable.

Key words: Denitrification, Metals contamination, Salt marshes, Ecosystem services, *Spartina maritima,* ^{15}N-isotope pairing technique

Introduction

In the last centuries, with increasing worldwide population in coastal areas, estuaries have been subdued to urban sewage, industrial discharges and agricultural runoff. These anthropogenic influences led to the increase in nutrients availability and consequently to eutrophication, named as "*cultural eutrophication*" (e.g. Hauxwell and Valiela, 2004; Howarth, 2008). The anthropogenic sources of metals into the aquatic systems have been reduced due to legal restrictive rules. However, metals contaminated sediments resulting from this "*historical contamination*" are still cause for concern due to their potential release into other

environmental matrices (Spencer et al., 2003; Vega et al., 2009). To our best knowledge, studies on denitrification in salt marshes are scarce (Valiela and Teal 1979; Erickson et al., 2003; Poulin et al., 2007). Combined effect of environmental stressors may affect estuarine communities, namely salt marsh services and the ecosystem health, and few authors have investigated the effect of metals on denitrification in salt marshes (Slater and Capone, 1984; Cao et al., 2008). Moreover, these studies were carried in North America marshes, respectively, Flax Pond (Long-Island, New York) and Carpinteria salt marsh (California), using the acetylene reduction method/acetylene inhibition technique.

Spartina maritima is one of the native halophytes colonizing European marshes. However, in some marshes this species has being replaced by the invasive *Spartina densiflora* (Poaceae) (e.g. Cambrollé et al., 2008). In other marshes, through an interspecific hybridization process with the introduced *Spartina alterniflora,* resulted in the fertile allopolyploid *Spartina anglica* (Ayres and Strong 2001), altering European marshes' species composition. As many marshes processes seem to be species-specific (e.g. Lillebø et al., 2006, Sousa et al., 2010a), it is important to address the services provided by this European native species. Thus, in the present study we hypothesize that denitrification, as a service provided by *Spartina maritima* marshes may be affected by the presence of metals, namely Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and the metalloid As. Denitrification was quantified in two monospecific *S. maritima* marshes differently stressed by metals, using the ^{15}N-isotope pairing technique to address the following questions: 1) does metal contamination on *Spartina maritima* salt marshes' rhizosediment affect denitrification?, 2) is denitrification, as an ecosystem service, increased or decreased in metal contaminated salt marshes, when compared to non-contaminated ones?

Material and methods

Sampling sites and procedure

According to the case study 3 in Chapter I, maximum denitrification in *S. maritima* marsh in a temperate estuary (Tagus estuary) occurs in winter. Thus, this was the chosen season to perform this study. In addition, two salt marshes, located in the southern European Atlantic margin (Portugal), with different historical contamination with metals were chosen: Tagus estuary (38^0 49' N, 08^0 56' W) and Mondego estuary (40^0 08'N, 08^0 50' W), representing, respectively the metal contaminated and the non-metal contaminated marsh. A detailed description of the systems can be seen in chapter 1 (case study 2, page 56). At each marsh, ten sediment cores (5 cm depth) were collected in spring tides during low tide, for sediment

characterization and for potential nitrification measurements. For denitrification quantification, other sediment cores (each one containing one or two shoots, with plant biomass as similar as possible) were collected using a Plexiglass core (∅ 8 cm; 30 cm height; sediment core with 15 cm depth). The cores were transported to the laboratory and immersed in estuarine water within 1h. Estuarine water was collected in containers and taken to the laboratory to be used in the incubation procedure. Temperature of sediment and water were recorded *in situ*.

Sediment characterization

Sediment particle size was determined by sequential sieving and classified according to Folk (1954). Organic matter was quantified as loss on ignition (% LOI) during 8 h at 500 ^{0}C. Microphytobenthos (MPB) Chlorophyll *a* was determined following the Lorenzen (1967) method. Total concentrations of metals in sediments were determined using 100 mg of freeze-dried aliquots, weighted into acid-washed Teflon bombs, with the addition of 1 mL of aqua regia and 6 mL of concentrated HF. The Teflon reactors were sealed and placed in a furnace for 1 hour at 100 ^{0}C. After cooling, the solution was quantitatively transferred into acid cleaned 100 mL volumetric flasks, which contained 5.6 g of boric acid (Loring and Rantala, 1990). The volume was completed with Milli-Q water and agitated to promote an easier dissolution of the boric acid, and the solution transferred to plastic bottles and stored at 4 ^{0}C. Metals were determined by ICP-MS (ICP-MS THERMO X Series, Peltier Nebulizing Camera, Burgener Nebulizer; CETAC AS510 auto-sampler; the CeO^{+}/Ce^{+} ratio was optimized at < 2 %; Internal standard: In).

In order to assess the accuracy and precision of the analytical methodology, analysis of certified reference materials were carried out (CRM - MESS-3 estuarine sediments), in parallel with samples. Certified and measured values were in agreement with recoveries between 60-90 %, average 71 %. All quantifications were done, at least, in triplicate and always with blank procedures run in parallel.

Potential nitrification quantification

Potential nitrification was measured according to Hansen (1980, in Rysgaard et al. 1994). A slurry incubation experiment was performed: 2 ml of homogenised surface sediment was incubated with 20 mM NH_4Cl and 4 mM KH_2PO_4 in 40 ml of artificial seawater (ASW). Salinity was adjusted to *in situ* values. Nitrification rates were measured through a time series experiment, thus quantifying hourly the NO_x-N (NO_3-N +NO_2-N) concentrations, over 5 h. Aliquots were centrifuged (8 min at 3000 rpm) and the supernatant sampled, filtered and immediately stored frozen for analysis. NO_x-N concentrations were expected to increase in a

linear way over the 5 hours, meaning that the added NH_4-N was nitrified immediately after the beginning of the incubation. Potential nitrification was calculated from this increase of NO_x-N and according to Rysgaard et al. (1994).

Incubation procedure – O_2 consumption

In the laboratory, the cores (∅ 8 cm, 30 cm height) were immersed in estuarine water in a tank/incubator and *in situ* light and temperature were simulated, in a batch mode assay, for each system. The samples were maintained aerated overnight (with an air pump and a magnetic stirrer rotating a magnet inside each core, as described at Cabrita and Brotas (2000) and Dalsgaard et al. (2000) and under natural seasonal light-dark cycles. On the following day, light and dark incubations were performed after sealing each core with plexiglass stoppers, and O_2 consumption calculated via mass balance.

Nitrogen and oxygen analyses

Water aliquots were filtered through GF/C Whatman filters and immediately stored frozen for quantification of inorganic nitrogen concentrations with a Tecator FIAstar_5000 Analyser. NO_3-N was quantified according to Grasshoff (1976), NO_2-N according to Bendschneider and Robison (1952), and NH_4-N according to Koroleff (1969/1970). Dissolved oxygen was quantified by Winkler titration (Grasshoff et al., 1983).

^{15}N- IPT assumptions

The assumptions of the IPT are the following: 1) the added $^{15}NO_3$ does not affect the production of $^{14}N_2$; 2) the produced $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ is binomial distributed; and 3) $^{14}NO_3$ and $^{15}NO_3$ mixes homogenously in the nitrate reduction zone in the sediment.

In order to ensure the ITP method assumptions a $^{15}NO_3$ concentration series experiment was performed following Nielsen (1992). Thus, seven different $^{15}NO_3$ concentrations (20-160 μM) were added to the overlying water column and denitrification rates were quantified.

Incubation procedure - Denitrification rates' quantification

Denitrification measurements were performed according to the isotope pairing technique (Nielsen, 1992). Light and dark incubations were performed separately but in the same cores as oxygen flux incubations. Vegetated sediment cores were immersed in estuarine water and $^{15}NO_3$ (from a $Na^{15}NO_3$ stock solution, 99% Sigma Aldrich) was added to the water container to a final concentration of at least 20% of the O_2 concentration in the incubation water. $^{15}NO_3$ diffusion time is dependent on the height of the sediment oxic layer. At time $T_{initial}$,

sediment cores were closed with plexiglass stoppers and incubation initiated. The incubation time was calculated based on the O_2 consumption data, so that the O_2 concentration never decreased 20% of the initial O_2. At time T_{final}, water aliquots were transferred to exetainer vials (Exetainer, Labco, High Wycombe, UK) for N_2 analyses. To immediately stop bacterial/any biological activity 200 µl of $ZnCl_2$ (50%, w/v) added to each exetainer. Water aliquots were treated as described in point 2.5 for NO_3 determination. Immediately after, each core was carefully mixed/slurried in order to homogenise the dissolved N_2 in the water column and in porewater and new aliquots for N_2 analyzes were collected. Finally, plants were washed and rinsed with distilled water and then dried at 60 ^{0}C until constant weight (about 48 h). Denitrification rates were calculated according to Nielsen (1992).

Statistical analysis

A principal components analysis (PCA) was performed using the PRIMER Version 5 software. The PCA is a technique used to identify patterns in data, expressing similarities and differences by projecting the data in a new coordinate system. In this projection, the greatest variance of the data comes to lie on the first coordinate (called the first principal component), the second greatest variance on the second coordinate, and so on. PCA can be used for dimensionality reduction without much loss of information. Projections considered the principal components 1 and 2 for environmental variables vectors: the incubation water, sediment characterization (data on Table 1) and metals concentrations, (data on Figure 1), and the study sites (contaminated and non-contaminated). All concentration data were log (x + 1) transformed. The following analyses were done with STATISTICA 9 software package. One-way ANOVA was performed to test for differences in potential nitrification rates between contaminated and non-metal-contaminated sites colonized by *Spartina maritima*. Cochran's Q and Kolmogorov-Smirnov tests were used to analyse homogeneity of variances and normality of data, respectively; and some data were transformed to satisfy the ANOVA assumptions. Two-way ANOVA was performed to test for differences in D_t (total denitrification) between marshes and between light/dark conditions. Linear correlation was performed (Pearson and Spearman rank correlations) to test for correlations between $^{15}NO_3$ concentration in the water column and D_w (D^{15}) and D_n (D^{14}).

Table 1 – Incubation water and sediment characterization for non-contaminated and contaminated salt marshes.

		Non-contaminated	Contaminated
Incubation water	Temperature (^{0}C)	17	18
	O_2	262 ± 11	221 ± 3
	NH_4-N	28.0 ± 8.8	22.8 ± 3.2
	NO_x-N	64.9 ± 13.3	43.2 ± 4.2
	Salinity (as *in situ*)	6 - 10	28 - 30
Sediment	*In situ* temperature (^{0}C)	12	16
	Granulometry	Fine sand: 37% (>125 μm) 13% (63-125μm) Silt and clay: 50% (<63μm)	Fine sand: 62% (>125 μm) 10% (63-125μm) Silt and clay: 28% (<63μm)
	LOI (%)	11.6 ± 0.4	13.5 ± 0.4
	MPB Chlo *a* (avrg±SD, n=5)	106.4 ± 18.1	46.4 ± 20.5
Plant (g DW/core) (avrg ± SD, n = 10)		2.2 ± 0.9	0.8 ± 0.5

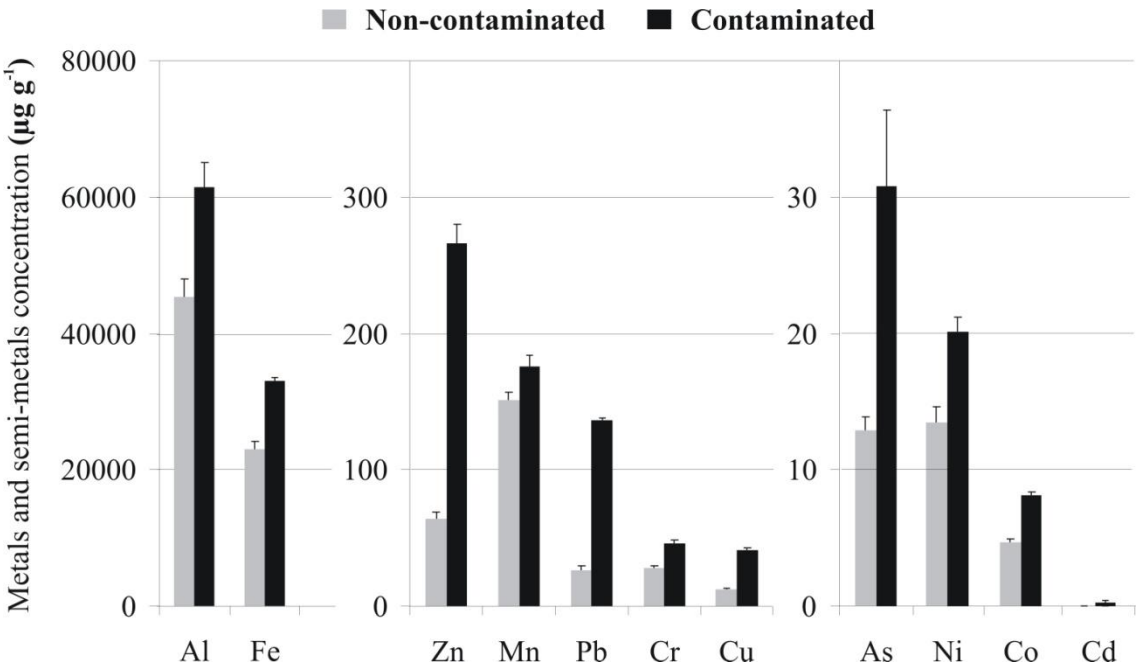


Figure 1. Concentration of metals (avrge±SD; n=3) Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and As in the sediment of the two studied *S. maritima* marshes.

Results

Study sites characterization

Concerning the concentrations of metals and semi-metals, the application of PCA showed that the principal components 1 and 2 explained 98.9 % of the variance. Figure 2 clearly shows that first principal component, which explains 95.1 % of the variance, separates to the right side of the axis (positive values) non-contaminated sediments (NC), and to the left side of the first principal component (axis negative values) contaminated sediments (C).

The second principal component explains 3.8 % of the variance and shows that there is some spatial variability within sites. Nevertheless, the PCA clearly shows that the two estuarine sediments differ in the degree of contamination of the metals Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and the metalloid As.

According to sediment quality guidelines proposed by Long et al. (1995), in the contaminated system, the concentrations of the metals Zn, Pb, Cu and As are above the ERL (effects range low: lowest concentration of a metal that produces adverse effects in 10 % of the data reviewed) and none of the metals has concentrations above the ERM (effects range median: level at which half of the studies reported harmful effects). However, it should be taken into account that these quality guidelines may not be considered as a threshold of sediment toxicity (O'Connor, 2004).

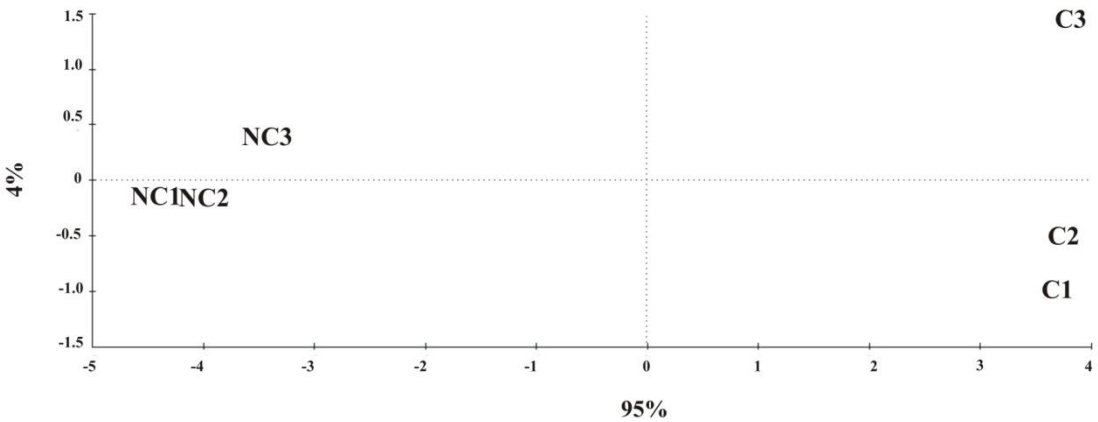


Figure 2. Results from the PCA, showing the principal components 1 and 2 for environmental variables vectors (the incubation water, sediment characterization metals and semimetal concentrations), and the study sites (non-contaminated and contaminated *S. maritima* salt marshes); NC = non-contaminated, C = contaminated.

Potential nitrification and Denitrification rates (non-contaminated vs contaminated salt marsh)

NO_x-N concentration increased over time in a linear way. Potential nitrification rates ranged between 169 ± 162 and 458 ± 189 nmol $N.cm^{-3}.h^{-1}$ (Figure 3a), and were not significantly different between non-contaminated salt marsh and contaminated salt marsh (One-way ANOVA, $F = 4.81$, $p > 0.05$) (Figure 3a).

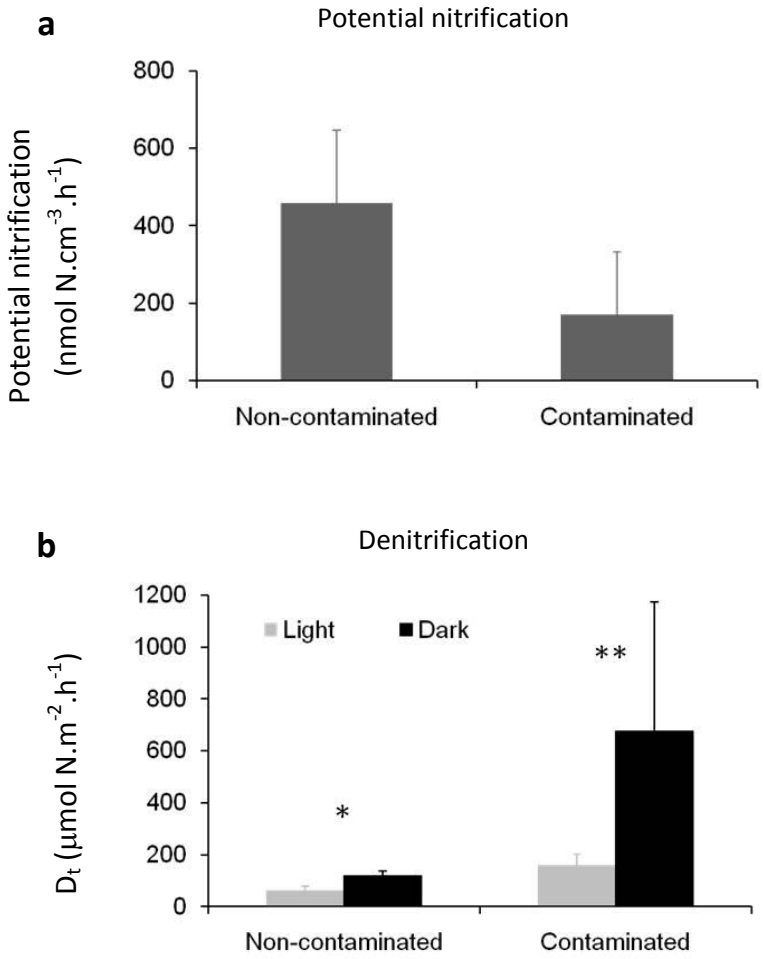


Figure 3. a) Potential nitrification (nmol $N.cm^{-3}.h^{-1}$; avrge ± SD, n=5), and b) Total denitrification (D_t) (µmol $N.m^{-2}.h^{-1}$; avrg ± SD, n=5) in dark and light conditions, comparing non-contaminated with contaminated *S. maritima* salt marshes.* means statistical significantly different ($p<0.05$).

Attending to the ^{15}N- IPT method assumptions, D_w (D^{15}; denitrification of bottom water NO_3^-) was significantly correlated to the $^{15}NO_3$ concentration in the water column ($p < 0.05$; $r = 0.9816$), while D_n (D^{14}; coupled nitrification-denitrification) was constant at all the $^{15}NO_3$ tested concentrations ($p > 0.05$; $r_s = 0.3907$). The results confirm that all assumptions of IPT were

fulfilled (Nielsen 1992, Rysgaard et al., 1994; Steingruber et al., 2001; Eyre et al., 2002), i.e. the method can be applied in this study.

Denitrification rates ranged between 60 ± 18 µmol N m^{-2} h^{-1} during daylight and 120 ± 17 µmol N m^{-2} h^{-1} during night at non-metal-contaminated *S. maritima* salt marsh and between 158 ± 44 µmol N m^{-2} h^{-1} during daylight and 676 ± 498 µmol N m^{-2} h^{-1} during night (Figure 3b) at the contaminated one. The first one had significantly lower denitrification rates (D_t) ($F = 21.63$, $p < 0.01$). In addition, denitrification was significantly lower under light conditions ($F = 13.43$, $p < 0.001$) (Table 2). Moreover, there was no interaction between light/dark incubations and the presence/absence of metal contamination in salt marshes. Regarding contribution of D_w (denitrification of bottom-water NO_3^-) and D_n (coupled nitrification-denitrification) to D_t, it was similar under dark conditions (i.e. around 50%) but during light incubations, D_n had a higher contribution (i.e. around 60%) to D_t than D_w (Table 2).

Table 2 – D_w, D_n and D_t (µmol N.m^{-2}.h^{-1}; avrg ± SD, n=5 at non-contaminated marsh and n=3 at contaminated marsh) in *Spartina maritima* vegetated sediment. Rates during light and dark conditions are indicated.

S. maritima salt marshes		**D_w**[a]	**D_n**[a]	**D_t**[a]
Light	Non-contaminated	25.3 ± 8.6	34.8 ± 9.5	60.1 ± 18.1
	Contaminated	56.5 ± 19.1	101.8 ± 24.9	158.3 ± 43.9
Dark	Non-contaminated	60.5 ± 11.0	59.8 ± 6.9	120.3 ± 17.0
	Contaminated	380.6 ± 296.6	295.3 ± 200.4	675.9 ± 497.0

[a]D_w - denitrification of bottom water NO_3^-; D_n - coupled nitrification-denitrification; D_t - total denitrification.

Considering, for the winter period, that days have 10 hours of light and 24 hours dark conditions, daily denitrification will be about 2285 ± 420 µmol N m^{-2} day^{-1} at the non-metal-contaminated *S. maritima* salt marsh and 11046 ± 7398 µmol N m^{-2} day^{-1} at the contaminated one. However, at the contaminated marsh the variability is much higher.

Discussion

Previous studies (e.g. Hu et al., 2002) have suggested that the presence of metals may inhibit nitrification processes, which may justify the lower potential nitrification at

contaminated sites. Moreover, a potentially higher D_n (coupled nitrification-denitrification) may be due to a higher nitrifier microbial community (Henrikson et al., 1981) at non-contaminated sites. Nevertheless, even though non-contaminated *S. maritima* marsh showed higher potential nitrification, differences are not statistically significant. This may be due to the high natural variability of estuarine systems (e.g. Erikson et al., 2003; Hou et al., 2007; Poulin et al., 2007), namely due to nutrient loadings, temperature, salinity, tidal influence, grain size, biotic disturbance, pollutants, which in turn affects the variability of the microbial community (Cao et al., 2006). Thus, potential nitrification can be affected by toxicity of metals. In turn, toxicity and availability of metals depend on abiotic/physico-chemical factors such as pH, cation exchange capacity, clay content, organic carbon, organic matter content (e.g. Teuchies et al., 2008; Du Laing et al., 2009), which determine toxicity to nitrifiers (O'Connor et al., 2004; Cao et al., 2008; Smolders et al., 2004).

In both systems (contaminated and non-contaminated *S. maritima* marsh) higher denitrification rates were observed during dark conditions. Higher nitrogen removal through denitrification during dark incubations may be related to the presence of the plants' roots; i.e. the microenvironments within the rhizosediment, which has been shown to enhance denitrification (e.g. Philipott et al., 2006; Philipott et al., 2009). In addition, under light conditions, microphytobenthos (MPB) will compete with microbial denitrifying community for NO_3 (Risgaard-Petersen et al. 2004; Hochard et al., 2010), leading to a lessening of denitrification. Thus, regarding the highest (more than 2 times) MPB concentration at non-contaminated sediment, denitrification in this salt marsh may be reduced when compared to the metal contaminated one. Besides nitrate concentration, total carbon or soluble organic carbon can also influence denitrification rates (Philipott et al., 2009). *S. maritima* contaminated marsh showed about 5 times higher denitrification rates than the non-contaminated marsh. By comparing the two studied marshes, it seems that the sediments with significantly higher concentrations of metals (Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and As) colonized by *S. maritima*, highly contribute to a higher denitrification activity and, consequently, to a higher N removal capacity converting it into dinitrogen gas (N_2). At least during winter and even having concentrations above the ERL for Zn, Pb, Cu and As (Long et al. 1995). Some studies have shown that denitrification activity is affected by metals contamination since denitrifier communities are metals sensitive (Slater and Capone, 1984; Probanza et al., 1996; Sakadevan et al., 1999; Holtan-Hartwig et al., 2002; Labbé et al., 2003; Magalhães et al., 2007; Pintathong et al., 2009). However, metals effect on denitrifier microbial community does not have a strict or unique response: other studies have shown that in the presence of certain metals, denitrification rates decrease in soils (sometimes depending

on metal concentrations) (Holtan-Hartwig et al., 2002) and wetland sediments (Sakadevan et al., 1999; Aigbavbiere, 2006; Magalhães et al., 2007), while Labbé et al. (2003) showed an increase in denitrification in a closed marine system (St. Lawrence Mesocosm at the Montréal Biodome, Canada). Accordingly, denitrification in *S. alterniflora* rhizosediment seemed to be increased by Pb, Cu, Zn, Cr addition, while Ni inhibited this process (Slater and Capone, 1984). In the present study, denitrifier microbial community seems to be stimulated and denitrification activity enhanced in the metal contaminated sediment. However, it should be taken into account that each metal has its own effect on denitrifier microbial community and enzymatic processes, and we are comparing sediments with a mixture of metals, i.e. denitrification in salt marshes differently stressed by mixture of metals. Thus, even though most of the mentioned studies show that metals contamination leads to a decrease in denitrification activity (the opposite of results obtained in the present work), these studies usually analyze the effect of each metal separately (Sakadevan et al., 1999; Yin et al., 2003; Aigbavbiere, 2006; Throbäck et al., 2007; Magalhães et al., 2007) rather than a mixture of metals, as occurs in "*historically contaminated systems*" (Bååth, 1989, in Probanza et al., 1996; Probanza et al., 1996; Holtan-Hartwig et al., 2002; this study). Moreover, sediment characteristics such as nutrient availability, organic matter content, grain size, also affect metal availability and speciation (Khan and Scullion, 2000; van Griethuysen et al., 2003; Magalhães et al., 2007; Reboreda and Caçador, 2007), which in turn affect denitrifier community. Thus, physico-chemical parameters of the sediment should also be considered when analyzing microbial activity and denitrification rates. In addition, microbial communities develop tolerance towards trace metals (Bååth, 1989, in Probanza et al., 1996; Cao et al., 2006; Sobolev and Begonia, 2008), which can influence its response to metals contamination, namely on denitrification activity.

To sum up, despite the higher variability, denitrification activity in the contaminated marsh is higher than at the non-contaminated one, which also results from an adaptation of *S. maritima* and nitrifiers community to the high metals concentrations in the sediment. However, other anthropogenic disturbances, e.g. habitat disruption and fragmentation, other pollutants or storm events may act as additional stressors. So, we may also ask, how resilient can these systems be? As stated by Boorman (2003) the relationship between marsh functions and environmental factors is complex and at present it is not possible to ascertain conclusions. Nevertheless, this study highlights that both potential nitrification and denitrification take place at sediments colonized by *S. maritima* contaminated with the metals Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and the metalloid As. Henceforth, more comparable results will be useful/valuable to evaluate the auto-remediation capacity of salt marshes through

denitrification, regarding multiple stressors, i.e. "*cultural eutrophication*" and "*historical contamination*" with metals. In addition, as *Spartina maritima* is an endangered native European species (Ayres and Strong 2001, Best et al., 2007, Cambrollé et al., 2008), these results also emphasize the negative consequences of an eventual disappearance of *S. maritima* salt marshes and its associated ecological services. Thus, these studies are essential for the management and restoration actions/plans in salt marshes, namely to enhance salt marshes auto-remediation capacity.

References

Eyre, B.D., Rysgaard, S., Dalsgaard, T., Christensen, P.B., 2002. Comparison of Isotope Pairing and N_2:Ar Methods for Measuring Sediment Denitrification – Assumptions, Modifications, and Implications. Estuaries 25, 1077-1067.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh capacity retention for heavy metals. Environmental Pollution 146, 147-154.

Aigbavbiere, E. E., 2006. The effects of heavy metals on denitrification in a wetland sediment. M.Sc thesis, Linköpings University, Sweden. http://www.essays.se/about/Ernest+Aigbavbiere/

Ayres, D.R., Strong, D.R., 2001. Origin and genetic diversity of *Spartina anglica* (Poaceae) using nuclear DNA markers. American Journal of Botany 88(10), 1863-1867.

Bendschneider, K., Robinson, R.J., 1952. A new spectrophotometric method for the determination of nitrite in sea water. Journal of Marine Research XI 1, 87-96.

Best, M., Massey, A., Prior A. 2007. Developing a saltmarsh classification tool for the European water framework directive. Marine Pollution Bulletin 55, 205–214.

Boorman, L.A., 2003. Saltmarsh Review. An overview of coastal saltmarshes, their dynamic and sensitivity characteristics for conservation and management. JNCC, Peterborough. On-line version at http://www.jncc.gov.uk/pdf/jncc334.pdf assessed at 30th July 2010.

Broos K., Warne M.St.J., Heemsbergen D.A., Stevens D., Barnes M.B., Correll R.L., McLaughlin M.J., 2007. Soil factors controlling the toxicity of copper and zinc to microbial processes in Australian soils. Environmental Toxicology and Chemistry, 26(4), 583–590.

Cabrita, M.T., Brotas, V., 2000. Seasonal variation in denitrification and dissolved nitrogen fluxes in intertidal sediments of the Tagus estuary. Marine Ecology Progress Series 202, 51–65.

Cambrollé, J., Redondo-Gómez, S., Mateos-Naranjo, E., Figueroa, M.E., 2008. Comparison of the role of two Spartina species in terms of phytostabilization and bioaccumulation of metals in the estuarine sediment. Marine Pollution Bulletin 56, 2037-2042.

Cao, Y., Cherr, G.N., Córdova-Kreylos, A.L., Fan, T.W.M., Green, P.G., Higashi, R.M., LaMontagne, M.G., Scow, K.M., Vines, C.A., Yuan, J., Holden, P.A., 2006. Relationships between sediment microbial communities and pollutants in two California salt marshes. Microbial Ecology 52, 619–633.

Cao, Y., Green, P.G., Holden P., 2008. Microbial Community Composition and Denitrifying Enzyme Activities in Salt Marsh Sediments. Applied and Environmental Microbiology 7585-7595.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O' Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 353-360.

Dalsgaard, T., Nielsen, L.P., Brotas, V., Viaroli, P., Underwood, G., Nedwell, D.B., Sundbäck, K., Rysgaard, S., Miles, A., Bartoli, M., Dong, L., Thornton, D.C.O., Ottosen, L.D.M., Castaldelli, G., Risgaard-Petersen, N., 2000. Protocol handbook for NICE - Nitrogen Cycling in Estuaries: A project under the EU research programme: Marine Science and Technology (MAST III). National Environmental Research Institute, Silkeborg, Denmark.

Du Laing, G., Rinklebe, J., Vandecasteele, B., Meers, E., Tack, F.M.G., 2009. Trace metal behaviour in estuarine and riverine floodplain soils and sediments: A review. Science of the Total Environment 407, 3972-3985.

Duffus, J., 2002. "Heavy Metals" – A Meaningless Term? (IUPAC Technical Report), Pure and Applied Chemistry 74(5), 793–807.

Eriksson, P.G., Svensson, J.M., Carrer, G.M., 2003. Temporal changes and spatial variation of soil oxygen consumption, nitrification and denitrification rates in a tidal salt marsh of the Lagoon of Venice, Italy. Estuarine, Coastal and Shelf Science 58, 861-871.

Folk, R.L., 1954. The distinction between grain size and mineral composition in sedimentary-rock nomenclature. Journal of Geology 62(4), 344–359.

Ghosh, M., Singh, S.P., 2005. A review on Phytoremediation of heavy metals and utilization of its byproducts. Applied Ecology and Environmental Research 3(1), 1-18.

Grasshoff, K. 1976. Methods of Seawater Analysis, Verlag Chemie, Weinheim.

Grasshoff, K., Ehrhardt, M., Kremling, K., 1983. Methods of seawater analysis. Verlag Chimie Weinheim, New York.

Hauxwell, J., Valiela, I., 2004. Effects of nutrient loading on shallow seagrass-dominated coastal systems: patterns and processes. In Nielsen, G. Banta, and M. Pedersen (eds) Estuarine Nutrient Cycling: The influence of Primary Producers. Kluwer Academic Publishers, London, p 59-92.

Henriksen, K., Hansen, J.I., Blackburn, T.H., 1981. Rates of nitrification, distribution of nitrifying bacteria, and nitrate fluxes in different types of sediment from Danish waters. Marine Biology 61, 299-304.

Hochard, S., Pinazo, C., Grenz, C., Evans, J.L.B., Pringault, O., 2010. Impact of microphytobenthos on the sediment biogeochemical cycles: A modeling approach. Ecological Modelling 221, 1687-1701.

Holtan-Hartwig, L., Bechmann, M., Høyas, T.R., Linjordet, R., Bakken, L.R., 2002. Heavy metals tolerance of soil denitrifying communities: N_2O dynamics. Soil Biology and Biochemistry 34, 1181-1190.

Hou, L.J., Liu, M., Xu, S.Y., Ou, D.N., Yu, J., Cheng, S.B., Lin, X., Yang, Y., 2007. The effects of semi-lunar spring and neap tidal change on nitrification, denitrification and N2O vertical distribution in the intertidal sediments of the Yangtze estuary, China. Estuarine, Coastal and Shelf Science 73, 607-616.

Howarth, R., 2008. Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmfull Algae 8, 14-20.

Hu, Z., Chandran, K., Grasso, D., Smets, B.F., 2002. Effect of nickel and cadmium speciation on nitrification inhibition. Environmental Science and Technology 36, 3074–3078.

Khan, M., Scullion, J., 2000. Effect of soil on microbial responses to metal contamination. Environmental Pollution 110, 115–125.

Koroleff, F., 1969/1970. Direct determination of ammonia in natural waters as indophenol blue. Int Counc Explor Sea (ICES) Comm Meet Pap 1969/C:9; revised 1970, 19–22.

Labbé, N., Parent, S., Villemur, R., 2003. Addition of trace metals increases denitrification rate in closed marine systems. Water Research 37, 914–920.

Lillebø, A.I., Flindt, M.R., Pardal M.A., Marques, J.C., 2006. The effect of *Zostera noltii, Spartina maritima* and *Scirpus maritimus* on sediment pore-water profiles, in a temperate intertidal estuary. Hydrobiologia, 555, 175-183.

Long, E.R., MacDonald, D.D., Smith, S.L., Calder, F.D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. Environmental Management 19, 81–97.

Lorenzen, C.J., 1967. Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography 12, 343–346.

Loring, D.H., Rantala, R.T.T., 1990. Techniques in Marine Environmental Sciences, 9, Sediments and Suspended particulate matter: Total and partial methods of digestion. International Council for the Exploration of the Sea, Copenhagen.

Magalhães, C., Costa, J., Teixeira, C., Bodalo, A.A., 2007. Impact of trace metals on denitrification in estuarine sediments of the Douro River estuary, Portugal. Marine Chemistry 107: 332–341.

Nielsen, L.P., 1992. Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiology Ecology 86, 357-362.

O'Connor, T.P., 2004. The sediment quality guideline, ERL, is not a chemical concentration at the threshold of sediment toxicity. Marine Pollution Bulletin 49, 383–385.

Oorts, K., Ghesquiere, U., Swinnen, K., Smolders, E., 2006. Soil properties affecting the toxicity of CuCl2 and NiCl2 for soil microbial processes in freshly spiked soils. Environmental Toxicology and Chemistry 25, 836–844.

Philipott, L., Hallin, S., Börjesson, G., Baggs, E.M., 2009. Biochemical cycling in the rhizosphere having an impact on global change. Plant and Soil 321(1-2), 61-81.

Philippott, L., Kufner, M., Chèneby, D., Depret, G., Laguerre, G., Martin-Laurent, F., 2006. Genetic structure and activity of the nitrate-reducers community in the rhizosphere of different cultivars of maize. Plant and Soil 287, 177–186.

Pintathong, P., Richardson, D.J., Spiro, S., Choorit, W., 2009. Influence of metal ions and organic carbons on denitrification activity of the halotolerant bacterium, *Paracoccus pantotrophus* P16 a strain from shrimp pond. Electronic Journal of Biotechnology 12(2), 1-11.

Poulin, P., Pelletier, E., Saint-Louis, R., 2007. Seasonal variability of denitrification efficiency in northern salt marshes: An example from the St. Lawrence Estuary. Marine Environmental Research 63, 490–505.

Probanza, A., Mañero, F.X.G., Ramos, B., Acero, N., Lucas, J.A., 1996. Effect of heavy metal son soil denitrification and CO_2 production after short term incubation. Microbiologia SEM 12, 417-424.

Risgaard-Petersen, N., Nicolaisen, M.H., Revsbech, N.P., Lomstein, B.A., 2004. Competition between ammonia-oxidizing bacteria and benthic microalgae. Applied Environmental Microbiology 70, 5528–5537.

Risgaard-Petersen, N., Rysgaard, S., Nielsen, L.P., Revsbech, N.P., 1994. Diurnal variation of denitrification and nitrification in sediments colonized by benthic microphytes. Limnology and Oceanography 39(3), 573–579.

Sakadevan, K., Zheng, H., Bavor, H.J. 1999. Impact of Heavy metals on denitrification in surface wetland sediments receiving wastewater. Water Science and Technology 40(3), 349-355.

Slater, J., Capone, D., 1986. Effects of metals on nitrogen fixation and denitrification in slurries of anoxic salt marsh sediment. Marine Ecology Progress Series 18, 89-95.

Smolders, E., Buekers, J., Oliver, I., McLaughlin, M.J., 2004. Soil properties affecting toxicity of zinc to soil microbial properties in laboratory-spiked and field-contaminated soils. Environmental Toxicology and Chemistry 23, 2633–2640.

Sobolev, D., Begonia, M.F.T., 2008. Effects of Heavy Metal Contamination upon Soil Microbes: Lead-induced Changes in General and Denitrifying Microbial Communities as Evidenced by Molecular Markers. International Journal of Environmental Research and Public Health 2008, *5(5)* 450-456.

Sousa A.I., Lillebø, A.I., Risgaard-Petersen, N., Pardal, M.A., Caçador, I., *in press*. Salt marshes' meaning on nitrogen remediation, In: Bioremediation: Biotechnology, Engineering and Environmental Management, Frank Columbus (chief ed.) Nova Science Publishers, Inc. NY, USA.

Sousa, A.I., Caçador, I., Lillebø, A.I., Pardal, M.A., 2008a. Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites. Chemosphere 70, 850–857.

Sousa, A.I., Lillebø, A.I., Caçador, I., Pardal, M.A., 2008b. Contribution of *Spartina maritima* to the reduction of eutrophication in estuarine systems. Environmental Pollution 156, 628–635.

Sousa, A.I., Lillebø, A.I., Pardal, M.A., Caçador, I., 2010a. Productivity and nutrient cycling in salt marshes: Contribution to ecosystem health. Estuarine, Coastal and Shelf Science 87, 640-646.

Spencer, K.L., Cundy, A.B., Croudace, I.W., 2003. Heavy metal distribution and earlydiagenesis in salt marsh sediments from the Medway Estuary, Kent, UK. Estuarine, Coastal and Shelf Science 57, 43–54.

Steingruber, S.M., Friedrich, J., Gachter, R., Wehrli, B., 2001. Measurement of denitrification in sediments with the ^{15}N isotope pairing technique. Applied Environmental Microbiology 67, 3771–3778.

Teuchies, J., de Deckere, E., Bervoets, L., Meynendonckx, J., van Regenmortel, S., Blust, R., Meire, P., 2008. Influence of tidal regime on the distribution of trace metals in a contaminated tidal freshwater marsh soil colonized with common reed (*Phragmites australis*). Environmental Pollution 155, 20-30.

Throbäck, I.N., Johansson, M., Rosenquist, M., Pell, M., Hansson, M., Hallin, S., 2007. Silver (Ag^+) reduces denitri¢cationand induces enrichmentof novel nirK genotypes in soil. FEMS Microbiology Letters 270, 189–194.

Valiela, I., Cole, M. L., Mcclelland, J., Hauxwell, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes. In M. P. Weinstein & D. A. Kreeger (Eds.), Concepts and Controversies in Tidal Marsh Ecology (pp. 23-38). Dordrecht, The Netherlands: Kluwer Academic Publishing.

Valiela, I., Teal, J.M., 1979. The nitrogen budget of a salt marsh ecosystem. Nature 280, 652-656.

van Griethuysen, C., Meijboom, E.W., Koelmans, A.A., 2003. Spatial variation of metals and acid volatile sulphide in floodplain lake sediment. Environmental Toxicology and Chemistry 22, 457–465.

Vega, F.A., Covelo, E.F., Reigosa, M.J., Andrade, M.L., 2009. Degradation of fuel oil in salt marsh soils affected by the Prestige oil spill. Journal of Hazardous Materials 166, 1020–1029.

Yin, S., Yang, L., Yin, B., Mei, L., 2003. Nitrification and denitrification activities of zinc-treated soils worked by the earthworm *Pheretima* sp. Biology and Fertility of Soils 38, 176–180.

GENERAL DISCUSSION

GENERAL DISCUSSION

Ecological meaning and ecosystem services provided by salt marshes have been widely discussed (Nixon, 1980; Costanza et al., 1997; Widdows and Brinsley, 2002; McLusky and Elliott, 2004). However, the way these services might be affected by multiple stressors is scarcely known. Accordingly, the main goals of the present thesis were to study in detail: 1) N cycling and N remediation capacity of warm-temperate salt marshes, as a way to counteract eutrophication, 2) metals' sequestration and compartmentalization in salt marsh plants, as phytoremediation and tolerance mechanisms in high metal contaminated salt marshes and 3) the effects of these multiple stressors (nitrogen enrichment and metals contamination) on the auto-remediation capacity of salt marsh plants and estuarine systems. The obtained results and knowleged concerning the salt marshes ecosystem services will be summarized and discussed in this section as an integrative approach.

Chapter I focuses on the N cycling in different compartments of salt marshes (plant biomass, detritus, litter, rhizosediment) and in different halophyte species. Productivity and nitrogen cycling of different salt marsh plants (Chapter I.1.) were shown to differ in their contribution to the N cycling and retention in these ecosystems. As previously observed in other systems (Sekiranda and Kiwanuka, 1998; Sollie and Verhoeven, 2008), salt marsh plants incorporate and retain N in their aboveground and belowground biomass. However, according to the present study, N pools and annual production in the aboveground and belowground plant material differ among species depending on their annual biomass production. In turn, biomass production depends on the physico-chemical characteristics of each species' rhizosphere. Salt marsh plants are known to increase their belowground biomass production under environmental stressing conditions such as flooding, high soil salinity and low nutrient availability (Ibañez et al., 1999; Scarton et al., 2002), which may explain the high belowground biomass production in some of the studied species. Plant belowground primary production, as well as the external sources, contribute to the N standing stocks in salt marshes' sediments, highlighting their role on N sequestration. Different plant species have different nitrogen cycling characteristics, and different N storage capacity in plant organs and in the sediment. Thus, zonation in the salt marsh (and inherent physico-chemical parameters), their annual biological cycle, phenology and physiology seem to influence the N retention/sequestration in salt marshes. However, their photosynthetic pathway does not seem to determine this ecosystem function since there is not a clear relationship between photosynthetic pathway and N retention efficiency. Therefore, photosynthetic pathway is not the only determinant

factor for the N use efficiency, since the studied C_4 species (*S. maritima*) is not always more efficient than C_3 species, counteracting previous studies' results (Jocic and Saric, 1983; Sage et al., 1987). As a whole, this work showed that the nitrogen uptake by salt marsh plants and its incorporation in biomass contribute to this nutrient cycling and sequestration - an important ecosystem service - and consequently, decrease its availability in the water column and potentially reduce eutrophication. Moreover, biomass production and nitrogen sequestration by salt marsh plants is a species-specific process, meaning that, although salt marsh species potentially share the same ecosystem services, their contribution for bioremediation seems to be species-specific.

Even though salt marsh plants have a crucial role on N sequestration, N cycling does not have the same pattern in *Spartina maritima* colonizing different salt marshes in different estuaries (Chapter I.2.). Biomass and N productions showed similar relative contribution of above- and belowground material to the total productions within each salt marsh, but it varied among the studied salt marshes. Belowground biomass and N productions increased from younger salt marshes to the oldest/mature one (young vs mature, defined by Valiela et al., 2000), wherein production showed the great amount of 96-97 % (the opposite occurred with the aboveground productions). *S. maritima* detritus N production and fate also varied according to the salt marsh: the oldest salt marsh showed the lowest detritus N production and younger ones showed the highest. However, detritus showed similar destinies in the salt marshes, with less than 5 % remaining near the plant and more than 95% of produced detritus being exported to adjacent areas of the salt marsh and the estuary. N budget annually stored within *S. maritima* rhizosediment is quite similar among younger salt marshes and it is 2 to 3 times higher in the oldest one. Accordingly, litter decomposition rates were higher in the younger salt marsh. Moreover, N accumulated in the rhizosediment is due to belowground production and external sources, demonstrating the sink capacity of these ecosystems. Thus, as recorded in other studies (Ibañez et al., 2000; Eyre and Ferguson, 2002; McGlathery et al., 2004), the ability to function/behave as a sink and/or source of N depends on the physico-chemical characteristics of the salt marsh, which in turn depends on the maturity of the salt marsh. Salt marsh physico-chemical parameters, such as salinity, flooding frequency, sediment type and drainage, strongly influence the plant biomass production (Ibañez et al., 2000; Curcó et al., 2002; Edwards and Mills, 2005). Urban sewage discharges to the Tagus estuary as well as inherent characteristics of an older/mature salt marsh, such as higher competition for nutrients, presence of more complex channels and subsequent modifications/reductions in the flooding frequency, might affect the nitrogen cycling as was observed in the oldest *S. maritima* salt marsh studied. As a whole, it can be concluded that rather than depending on the estuary,

S. maritima N cycling depends on the salt marshes characteristics. To sum up, N cycling in *S. maritima* salt marshes is not specific for an estuary. It rather depends on the salt marshes' biotic and abiotic parameters. Therefore, the *S. maritima* sink capacity for N (different plant compartments - plant organs/detritus/litter – and rhizosediment) was higher in the oldest salt marsh, which means that this salt marsh potentially has a higher capacity to ameliorate eutrophication through N incorporation in biomass and its sequestration within the estuarine sediment.

S. maritima salt marshes have a potentially higher contribution to the reduction of available nitrogen in winter, through denitrification (Chapter I.3). At this season the competition between primary producers (*S. maritima* and MPB) and nitrifiers is lower. This study shows that *S. martima* marshes contributes to the reduction of the land-driven nitrogen loading to the open ocean, however, on an annual basis it cannot be stated that it is significantly different from the sediments without vegetation. Nevertheless, the significantly higher contribution *S. martima* marshes in N removal during winter contributes to the reduction in nitrate availability in the following spring.

Considering the metals contamination of salt marshes during the last centuries, known as historical contamination (Vega et al., 2009) and the salt marsh plants capacity to tolerate and sequestrate metals (Matthews et al., 2005), **Chapter II** focuses on *H. portulacoides* metals accumulation in different cell compartments and cell organs, as a phytoremediation process. *H. portulacoides* up took metals (Zn, Pb, Co, Cd, Ni and Cu) and retained them mostly in the roots, rather than in the aboveground organs (leaves and stems), as also recorded in other works and/or plant species (Stoltz and Greger, 2002, Fitzgerald et al., 2003; Matthews et al., 2004; Fritioff and Greger, 2006; Reboreda and Caçador, 2007; Caçador et al., 2009). This may be related to the low mobility of the metals once inside the plant (Deng et al., 2004). Regarding the metal location in cell compartments, this study demonstrated that *H. portulacoides* mostly retain metals in the cell wall (53 % in leaves to 65 % in roots) and the metal content in the intracellular compartment is much lower (21% in roots to 32% in leaves). Compartmentation of metals outside key metabolic sites in the *H. portulacoides*' cells, as occurs in other plant species (Rauser and Ackerley, 1987; Lozano-Rodriguez et al., 1997; Küpper et al., 2001; Psaras and Manetas, 2001; Carrier et al., 2003), might be a tolerance mechanism of this plant to high metals concentrations in the rhizosediment.

Multiple stressors, particularly cultural eutrophication (Chapter I) and metals historical contamination (Chapter II), and its effects on the salt marsh plants phytoremediation capacity was evaluated on **Chapter III**.

According to the results of Chapter III.1., *H. portulacoides* can uptake and sequestrate high metal concentrations (phytoaccumulation) and roots may act as a barrier for translocation of Zn, Cu, Cd from roots to leaves, preventing toxicity as reported in Chapter II.1 (Sousa et al., 2008) and in other works (e.g. Windham et al., 2003; Weis and Weis, 2004; Quan et al., 2007; Bose et al., 2008; Caçador et al., 2009; Bonanno and Giudice, 2010). Micronutrient metals (Zn, Cu, Ni) uptake by *H. portulacoides* does not seem to depend on the nitrogen availability neither, in the case of Zn, on the time of exposure (at least for 6 months). The nitrogen enrichment on the *H. portulacoides* rhizosediment, at least for the tested concentrations, does not affect its ability to sequestrate Zn, Cu and Ni through phytoaccumulation. Moreover, after 6 months of exposure, Cu and Ni uptake by *H. portulacoides* increased and was phytoaccumulated in roots, reflecting this plant ability to adapt and increase its tolerance and sequestration capacity for these metals. As occurs in other species (Deng et al., 2004; Bose et al., 2008), nitrogen availability in the sediment may not be the main factor determining the Zn, Cu and Ni uptake by *H. portulacoides*. In the present study, multiple stressors (cultural eutrophication and historical contamination with Zn, Cu, Ni and Cd) do not affect the *H. portulacoides* phytoremediation capacity for Zn, Cu and Ni. In contrast, the increase of tested N concentrations may enhance the Cd phytoaccumulation, which means that this ecosystem service is promoted by cultural eutrophication. Nevertheless, since Cd is a priority substance under the Water Framework Directive legislation (Annex II, Directive 2008/105/EC) (http://ec.europa.eu/environment/water/water-dangersub), is very toxic and is bioaccumulated throughout the food web, its direct and indirect effects as a pollutant and salt marshes vulnerability (Best et al., 2007) should be taken into account. Accordingly, salt marshes reduction will have drastic consequences to the ecosystem and human population.

The work reported in Chapter III.2 evaluates the auto-remediation capacity of salt marshes through denitrification, regarding multiple stressors, *e.g.* "*cultural eutrophication*" and "*historical contamination*" with metals. In winter, daily denitrification was about 2285 ± 420 µmol N m^{-2} day^{-1} at the non-metal-contaminated *S. maritima* marsh and 11046 ± 7398 µmol N m^{-2} day^{-1} at the contaminated one, which may be due to an adaptation of *S. maritima* and nitrifiers community to the high metals concentrations in the sediment. However, due to the high variability obtained, more comparable results will be valuable. Regarding *Spartina maritima* classification as an endangered native European species (Ayres and Strong, 2001; Best et al., 2007; Cambrollé et al., 2008), these results also emphasize the negative consequences of an eventual disappearance of *S. maritima* salt marshes, as previously mentioned by Quan et al., (2007), regarding replacement of the native species *Phragmites*

australis and *Scirpus mariqueter* by *Spartina alterniflora*. Thus, these studies are essential for the management and restoration plans in salt marshes, namely to enhance salt marshes auto-remediation capacity.

As a whole, the present thesis highlights the crucial role of salt marshes on the potential reduction/mitigation of eutrophication through nitrogen cycling and sequestration. Although within the same system there may be some spatial variation. The native European salt marsh species *Spartina maritima* contributes to nitrogen remediation by intercepting the land-derived nitrogen and buffering the loading of reactive nitrogen to the open ocean through nitrogen incorporation in biomass, organic nitrogen burial and denitrification. In addition, salt marsh plants ability to accumulate high metals concentrations and inherently "clean" (phytoremediate) the surrounding estuarine environment was recorded. Lastly, the studied multiple stressors, nitrogen loading and metals contamination, did not affect the phytoremediation capacity of *H. portulacoides* for Zn, Cu and Ni; in contrast, it enhanced the Cd accumulation in this plant species. However, Cd toxicity and bioaccumulation throughout the foodweb as well as salt marshes vulnerability should not be forgotten. Denitrification in metals-contaminated (Al, Fe, Zn, Mn, Pb, Cr, Cu, Ni, Co, Cd and the metalloid As) salt marsh was higher, during the studied season (winter), when compared to a non-contaminated salt marsh. This suggests adaptation of *S. maritima* and nitrifiers microbial community to the high metals concentrations in the sediment. Nevertheless, more comparable results will be valuable, namely in other seasons. The relationship between marsh functions and environmental factors is complex (Boorman, 2003; Day et al., 2008). However, the described ecosystem processes represent salt marsh services, which should gain greater importance considering main anthropogenic threats to these coastal areas, i.e. the reduction or fragmentation of salt marsh areas (e.g. Best et al., 2007; Gedan et al., 2009), the increasing of the land-derived nitrogen loading (e.g. Gruber and Galloway, 2008; Philipott et al., 2009) and metal contamination, species introduction and/or invasive species; as well as the consequences of global climate change that our planet and all its ecosystems are facing (e.g. CO_2 increase, temperature increase, sea level rise) (Day et al., 2008; Gedan et al., 2009). In addition, as a result of interactions with other human activities in coastal regions, climate change effects may become even more severe (e.g., Pont et al. 2002). Actually, when multiple stressors affect salt marsh and/or wetland plants, their capacity to adapt to new stressing is reduced (Mendelssohn and Morris, 2000, this thesis). Management and recovery of salt marsh services that had been lost or affected by anthropogenic influence or as a consequence of other external agent might be performed as an integrative approach, thus taking into account all the ecosystem services

involved (Gedan et al., 2009). According to Mateos-Naranjo et al. (2010), *S. maritima* salt marshes, which will be likely affected by these climate scenarios' effects (namely increasing atmospheric CO_2 concentration and higher salinities), will increase their productivity and consequently promote their ecosystem functions. Moreover, carbon sink role of salt marshes (e.g. Caçador et al., 2004; Sousa et al., 2010) will contribute to counteract this increase in atmospheric CO_2 concentration and help to prevent salt marshes from fragmentation and/or disruption and threat of their ecosystem functions and services.

Since *Spartina maritima* is an endangered native European species (Ayres and Strong 2001; Best et al., 2007; Cambrollé et al., 2008), the results of this thesis emphasize the negative consequences of the eventual disappearance of *S. maritima* salt marshes and its associated ecological services. The outcome of this work is therefore a valuable tool to be considered in future management and restoration plans of salt marshes. As a whole, multiple stressors affected the auto-remediation capacity of salt marshes. Given that ecosystem functions seem to be species-specific, one cannot exclude that multiple stressors threaten the provided ecosystem services and, consequently, ecosystem health and equilibrium may be endangered.

REFERENCES

Ayres, D.R., Strong, D.R., 2001. Origin and genetic diversity of *Spartina anglica* (Poaceae) using nuclear DNA markers. American Journal of Botany 88(10), 1863-1867.

Best, M., Massey, A., Prior A. 2007. Developing a saltmarsh classification tool for the European water framework directive. Marine Pollution Bulletin 55, 205–214.

Bonanno, G., Giudice, R.L., 2010. Heavy metal bioaccumulation by the organs of *Phragmites australis* (common reed) and their potential use as contamination indicators. Ecological Indicators 10, 639-645.

Boorman, L.A., 2003. Saltmarsh Review. An overview of coastal saltmarshes, their dynamic and sensitivity characteristics for conservation and management. JNCC, Peterborough. On-line version at http://www.jncc.gov.uk/pdf/jncc334.pdf assessed at 30th July 2010.

Bose, S.,Vedamati, J., Rai, V., Ramanathan, A.L., 2008. Metal uptake and transport by *Typha angustata* L. grown on metal contaminated waste amended soil: An implication of phytoremediation. Geoderma 145, 136-142.

Caçador, I., Caetano, M., Duarte, B., Vale, C., 2009. Stock and losses of trace metals from salt marsh plants. Marine Environmental Research 67, 75-82.

Caçador, I., Costa, A.L., Vale, C., 2004. Carbon storage in Tagus salt marsh sediments. Water, Air, and Soil Pollution , 701-714.

Cambrollé, J., Redondo-Gómez, S., Mateos-Naranjo, E., Figueroa, M.E., 2008. Comparison of the role of two Spartina species in terms of phytostabilization and bioaccumulation of metals in the estuarine sediment. Marine Pollution Bulletin 56, 2037-2042.

Carrier, P., Baryla, A., Havaux M., 2003. Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contaminated soil. Planta 216, 939–950.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O' Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., van den Belt, M., 1997. The value of the world's ecosystem services and natural capital. Nature 387, 353-360.

Curcó, A., Ibañez, C., Day, J.W., Prat, N., 2002. Net primary production and decomposition of salt marshes of the Ebre Delta (Catalonia, Spain). Estuaries 25, 309-324.

Day, J.D., Christian, R.R., Boesch, D.M., Yáñez-Arancibia, A., Morris J., Twilley, R.R., Naylor, Schaffner, L., Stevenson, C, 2008. Consequences of Climate Change on the ecogeomorphology of Coastal Wetlands. Estuaries and Coasts 31, 477–491.

Deng, H., Yea, Z.H., Wong, M.H., 2004. Accumulation of lead, zinc, copper and cadmium by 12 wetland plant species thriving in metal-contaminated sites in China. Environmental Pollution 132, 29-40.

Edwards, K.R., Mills, K.P., 2005. Aboveground and belowground productivity of *Spartina arteniflora* (smooth cordgrass) in natural and created Louisiana salt marshes. Estuaries 28, 252-265.

Eyre, B.D., Ferguson, A.J.P., 2002. Comparison of carbon production and decomposition, benthic nutrient fluxes and denitrification in seagrass, phytoplankton, benthic microalgae- and macroalgae-dominated warm-temperate Australian lagoons. Marine Ecology Progress Series 229, 43-59.

Fitzgerald, E.J., Caffrey, J.M., Nesaratnam, S.T., McLoughlin, P., 2003. Copper and lead concentrations in salt marsh plants on the suir Estuary, Ireland. Environmental Pollution 123, 67-74.

Fritioff A, Greger M. 2006. Uptake and distribution of Zn, Cu, Cd, and Pb in an aquatic plant *Potamogeton natans* . Chemosphere 63(2), 220–227.

Gedan, K.B., Silliman, B.R., Bertness, M.D., 2009. Centuries of Human-Driven Change in Salt Marsh Ecosystems. Annual Review Marine Science 1, 117-141.

Gruber, N., Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451, 293-296.

Ibañez, C., Curcó, A., Day Jr, J.W., Prat, N. 2000. Structure and productivity of microtidal Mediterranean coastal marshes, in: Weinstein M.P., Kreeger, D.A., (Eds.), Concepts and Controversies in Tidal Marsh Ecology. Kluwer Academic Publishers, Netherlands, pp. 107-136.

Ibañez, C., Day, Jr.J.W., Pont, D. 1999. Primary production and decomposition of wetlands of the Rhône Delta, France: interactive impacts of human modifications and relative sea level rise. Journal of Coastal Research 15, 717–731.

Jocic, B., Saric, M.R., 1983. Efficiency of nitrogen, phosphorus, and potassium use by corn, sunflower, and sugarbeet for the synthesis of organic matter. Plant and Soil 72, 219-223.

Küpper, H., Lombi, E., Zhao, F., Wieshammer, G., McGrath, S.P., 2001. Cellular compartmentation of nickel in the hyperaccumulators Alyssum lesbiacum, Alyssum bertolonii and Thlaspi goesingense. J. Exp. Bot. 52, 2291–2300.

Lozano-Rodriguez, E., Hernandéz, L.E., Bonay, P., Carpena-Ruiz, R.O., 1997. Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. Journal of Experimental Botany 48, 123-128.

Mateos-Naranjo, E., Redondo-Gómez, S. Andrades-Moreno, L., Davy, A.J., 2010. Growth and photosynthetic responses of the cordgrass *Spartina maritima* to CO2 enrichment and salinity. Chemosphere. doi:10.1016/j.chemosphere.2010.07.047

Matthews, D.J., Moran, B.M., McCabe, P.F., Otte, M.L., 2004. Zinc tolerance, uptake, accumulation and distribution in plants and protoplasts of five European populations of the wetland grass *Glyceria fluitans*. Aquat. Bot. 80, 39–52.

Matthews, D.J., Moran, B.M., Otte, M.L., 2005. Screening the wetland plant species Alisma plantago-aquatica, Carex rostrata and Phalaris arundinacea for innate tolerance to zinc and comparison with Eriophorum angustifolium and Festuca rubra merlin. Environmental Pollution 134, 343–351.

McGlathery, KJ., Sundbäck, K., Anderson, I.C., 2004. The importance of primary producers for benthic nitrogen and phosphorus cycling, in: Nielsen, S., Banta, G., Pedersen, M., (Eds.), Estuarine nutrient cycling: The influence of primary producers. Kluwer Academic Publishers, The Netherlands, pp. 231-261.

McLusky, D.S., Elliot, M., 2004. The Estuarine Ecosystem - Ecology, Threats, and Management, 3rd ed. Oxford University Press.

Mendelssohn, I.A., Morris, J.T. 2000. Eco-physiological controls on the productivity of Spartina alterniflora Loisel. Pp. 59-80. In: Concepts and Controversies in Tidal Marsh Ecology, M. Weinstein (ed) Kluwer Press, Boston.

Nixon, S.W., 1980. Between coastal marshes and coastal waters-a review of twenty years of speculation and research on the role of salt marshes in estuarine productivity and water chemistry, in: Hamilton, P. Macdonald, K.B., (Eds.), Estuarine and Wetland Processes with Emphasis on Modelling. Plenum Press, New York, pp. 437-525.

Philipott, L., Hallin, S., Börjesson, G., Baggs, E.M., 2009. Biochemical cycling in the rhizosphere having an impact on global change. Plant and Soil 321(1-2), 61-81.

Pont, D., J. Day, P. Hensel, E. Franquet, F. Torre, P. Rioual, C. Ibañez, and E. Coulet. 2002. Response scenarios for the deltaic plain of the Rhône in the face of an acceleration in the rate of sea level rise, with a special attention for Salicornia-type environments. Estuaries 25: 337–358.

Psaras, G.K., Manetas, Y., 2001. Nickel localization in seeds of the metal hyperaccumulator Thlaspi pindicum Hausskn. Ann. Bot. (London) 88, 513–516.

Quan, W.M., Han, J.D., Shen, A.L., Ping, X.Y., Qian, P.L., Li, C.J., Shi, L.Y., Chen, Y.Q., 2007. Uptake and distribution of N, P and heavy metals in three dominant salt marsh macrophytes from Yangtze River estuary, China. Marine Environmental Research 64, 21–37.

Rauser, W.E., Ackerley, C.A., 1987. Localization of cadmium in granules within differentiating and mature root cells. Can. J. Bot. 65, 643–646.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh capacity retention for heavy metals. Environmental Pollution 146, 147-154.

Sage, R.F., Pearcy, R.W., Seemann, J.R., 1987. The Nitrogen Use Efficiency of C3 and C4 Plants. III. Leaf nitrogen effects on the activity of carboxylating enzymes in *Chenopodium album* (L.) and *Amaranthus retroflexus* (L.). Plant Physiology 85, 355-359.

Scarton, F., Day, J.W., Rismondo, A., 2002. Primary Production and Decomposition of *Sarcocornia fruticosa* (L.) Scott and *Phragmites australis* Trin. Ex Steudel in the Po Delta, Italy. Estuaries 25, 325-336.

Sekiranda, S.B.K., Kiwanuka, S., 1998. A study of nutrient removal efficiency of Phragmites mauritianus in experimental reactors in Uganda. Hydrobiologia 364, 83-91.

Sollie, S., Verhoeven, J.T.A., 2008. Nutrient cycling and retention along a Littoral Gradient in a Dutch shallow Lake in relation to water level Regime. Water, Air and Soil Pollution 193, 107-121.

Sousa A.I., Lillebø A.I , Pardal M.A., Caçador I. (2010) The influence of *Spartina maritima* on carbon retention capacity in salt marshes from warm-temperate estuaries. Marine Pollution Bulletin 61: 215-223.

Sousa, A.I., Caçador, I., Lillebø, A.I., Pardal, M.A., 2008. Heavy metal accumulation in *Halimione portulacoides*: intra- and extra-cellular metal binding sites. Chemosphere 70, 850–857.

Stoltz, E., Greger, M., 2002. Accumulation properties of As, Cd, Cu, Pb and Zn by four wetland plant species growing on submerged mine tailings. Environmental and Experimental Botany 47, 271-280.

Valiela, I., Cole, M. L., Mcclelland, J., Hauxwell, J., Cebrian, J., Joye, S.B., 2000. Role of salt marshes as part of coastal landscapes. In M. P. Weinstein & D. A. Kreeger (Eds.), Concepts and Controversies in Tidal Marsh Ecology (pp. 23-38). Dordrecht, The Netherlands: Kluwer Academic Publishing.

Vega, F.A., Covelo, E.F., Reigosa, M.J., Andrade, M.L., 2009. Degradation of fuel oil in salt marsh soils affected by the Prestige oil spill. Journal of Hazardous Materials 166, 1020–1029.

Weis, J.S., Weis, P., 2004. Metal uptake, transport and release by wetland plants: implications for phytoremediation and restoration. Environmental International 30, 685-700.

Widdows, J., Brinsley, M., 2002. Impact of biotic and abiotic processes on sediment dynamics and the consequences to the structure and functioning of the intertidal zone. Journal of Sea Research 48, 143-156.

Windham, L, Weis, J.S., Weis, P., 2003. Uptake and distribution of metals in two dominant salt marsh macrophytes, *Spartina alterniflora* (cordgrass) and *Phragmites australis* (common reed). Estuarine, Coastal and Shelf Science 56, 63–72.

FUTURE PERSPECTIVES

All the work reported in this thesis allowed to assess the majority of the questions proposed in the beginning of the work. Nevertheless, there are still some features that could be better understood. For instance, potential nitrification was higher on metal contaminated sediments, rather than on non-contaminated ones. It was proposed that it might be an adaptation of nitrifiers community to high metal concentrations. Now, the pertinent question is: are all the metals likewise responsible for this higher potential nitrification, or does it depend on the metals? Does this effect depend on the metals concentrations?

Denitrification rates' measurements in this study were highly variable, as has been documented in other works. Thus, the reduction of the variability may be an important step for the future. Regarding the limitations within each method for quantification of denitrification, it becomes difficult to compare different works. Thus, one challenge that arises is to perform future studies in order to obtain more comparable results.

Lastly, in a scenario of global climate change that all ecosystems are facing nowadays (and inherent sea level rise, temperature increase, atmospheric CO_2 increase, etc.), what would be the effects of eventual reduction, fragmentation and/or disappearance of salt marshes on the global ecosystem health and equilibrium, as well as on the services provided by these ecosystems, namely nutrient cycling and its contribution to reduce eutrophication and phytoremediation?

ACKNOWLEDGEMENTS/AGRADECIMENTOS (PT)

Esta dissertação representa o culminar de mais uma etapa, cuja realização não teria sido possível sem a ajuda de várias pessoas, às quais gostaria de manifestar o meu agradecimento.

Aos meus orientadores, Prof. Doutora Isabel Caçador, Doutora Ana Isabel Lillebø e Prof. Doutor Miguel Pardal pela permanente disponibilidade, pela sua amizade, por todo o apoio científico prestado, e por toda a força que me transmitiram, factores imprescindíveis para a realização desta dissertação.

To Dr. Nils Risgaard-Petersen, for promptly receiving me in his laboratory and for all the scientific guidance regarding the ^{15}N-IPT. Thank you also for helping me to solve all denitrification "troubles"!

À Prof. Doutora Vanda Brotas, por ter prontamente disponibilizado equipamento essencial à concretização deste trabalho.

A todos os meus colegas e amigos do Instituto de Oceanografia (IO), pela sua boa disposição, amizade e por toda a ajuda prestadas ao longo deste período, nomeadamente: Bruno Jesus, Paulo Cartaxana, Lourenço Ribeiro, Carla Gameiro, Filipe Neves, Alberto Azevedo, Marta Delgado, Tânia Diniz, Bernardo Duarte, Tadeu Pereira, Sílvia Pedro, Paula Chaínho, Luísa Chaves, João Paulo, Nuno Prista (a estes dois últimos agradeço especialmente pela companhia fora de horas no IO!).

À D. Manuela Lucas, pela sua amizade, constante disponibilidade e ajuda em todas tarefas laboratoriais!

A todos os meus colegas e amigos do IMAR - Coimbra por me terem feito sentir ainda em casa aquando das minhas visitas a Coimbra: Filipe Martinho, Sara Leston, Gabi (sempre entusiasta e disponível), Patrícia Cardoso, Sónia Cotrim, Joana Oliveira, Alexandra Baeta,…(desculpem se me estiver a esquecer de alguém!)

A todos aqueles amigos que me acompanharam nas saídas de campo, sem os quais a concretização deste trabalho não teria sido possível!

A todos os meus amigos que sempre estiveram presentes nos momentos certos, pelo incentivo e acima de tudo pela sua amizade: Carolina Sá, Helena Coelho, Joana Salgado,

Andreia Caseiro, Patrícia Fonseca, Hélder Leong, Sérgio Muacho, Rosa Reboreda e outros, dos quais posso não me estar a lembrar agora….

Aos meus pais, Adelina e Carlos, que sempre me apoiaram e proporcionaram a concretização dos meus sonhos.

À minha irmã, Ana Luísa, pelo apoio incondicional que sempre me deu.

A todos muito obrigada!

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR Portugal

Este trabalho foi financiado através de uma Bolsa de Doutoramento (SFRH/BD/23634/2005) concedida pela Fundação para a Ciência e a Tecnologia.